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N. F. XI

FIRST INTERIM REVISION ANNOUNCEMENT

National Formulary

Eleventh Edition

OFFICIAL FROM JANUARY 1, 1961*

*By virtue of the authority conveyed in Chapter III, Article X and Chapter IX, Article V of the By-Laws of the American Pharmaceutical Association, the Council of the Association appointed the Committee on National Formulary, approved the text of this Interim Revision Announcement prepared by the Committee, authorized the printing thereof, and fixed January 1, 1961, as the date upon which it shall become official**.

WILLIAM S APPLE, *Secretary*
Council of the A. Ph. A.

EDWARD G. FELDMANN, *Chairman*
Committee on National Formulary

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* Monographs on Vitamin B_{12} with Intrinsic Factor Concentrate, Vitamin B_{12} with Intrinsic Factor Concentrate Capsules, and Vitamin B_{12} with Intrinsic Factor Concentrate Tablets, and the section on Antianemia Preparations are effective from January 1, 1961.
Other items included in this Interim Revision Announcement may be considered as effective immediately upon publication.

Additions, Changes, and Corrections

(Page numbers cited refer to N. F. XI, except where otherwise indicated)

General Notices, page 5

Change the second line of the paragraph entitled *Odorless* to read:

. . .*Description* applies to examination, after exposure to the air for 15 minutes, of a. . .

General Notices, Light-Resistant Container, page 6

Postpone the effectiveness of the entire second paragraph until further notice.

Exsiccated Alum, page 19

Change the first paragraph in the monograph to read:

Exsiccated Alum, dried at 200° for 4 hours, contains not less than 96.5 per cent of $AlNH_4(SO_4)_2$ or not less than 96.5 per cent and not more than 105 per cent of $AlK(SO_4)_2$.

Dibasic Amphetamine Phosphate, page 26

In the paragraph entitled *Residue on ignition*, change the page reference in the third line to read:

. . .page 25.

Beef, Iron and Wine, page 42

Change the last sentence in the monograph to read:

One usual dose contains 300 mg. of Beef Extract and 500 mg. of Ferric Ammonium Citrate.

Dibasic Calcium Phosphate, page 68

Change the first sentence in the paragraph entitled *Assay*, page 69, to read:

Assay—Dissolve about 300 mg. of Dibasic Calcium Phosphate, previously ignited at 800° to 825° to constant weight and accurately weighed, in 10 ml. of diluted hydrochloric acid, add about 120 ml. of water, and heat almost to boiling."

Magnesium Citrate Solution, page 195

In the second line of the formula for Magnesium Citrate Solution, change Citric Acid to read:

Anhydrous Citric Acid. . . . . 27.4 Gm.

Following the formula for Magnesium Citrate Solution, page 195, insert a note to read:

NOTE: An amount of citric acid containing 1 molecule of water of hydration, equivalent to 27.4 Gm. of anhydrous citric acid, may be used in the above formula.

Tribasic Magnesium Phosphate, page 197

Change the first paragraph in the monograph to read:

Tribasic Magnesium Phosphate, ignited to constant weight, contains not less than 98 per cent and not more than 101.5 per cent of $Mg_3(PO_4)_2$.

Phenolphthalein, page 276

In the third line of the paragraph entitled *Color of solution*, change the word Watching to read:

Matching. . . .

Sodium Acetate, page 328

Change the first paragraph of the monograph to read:

Sodium Acetate, dried at 80° overnight and followed by drying at 120° for 4 hours, contains not less than 99 per cent and not more than 101.0 per cent of $C_2H_3NaO_2$, calculated on the anhydrous basis.

Vitamin B_{12} with Intrinsic Factor Concentrate, page 392

Change the monograph to read:

VITAMIN B_{12} WITH INTRINSIC FACTOR CONCENTRATE

Vitamin B_{12} with Intrinsic Factor Concentrate possesses vitamin B_{12} activity made more readily absorbable from the gastrointestinal tract of patients suffering from pernicious anemia by combination with suitable preparations of the stomach or intestine of domestic animals used for food by man. Each N. F. Unit of Vitamin B_{12} with Intrinsic Factor Concentrate contains 10 mcg. of cobalamins, calculated as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), with the amount, not to exceed 300 mg., of intrinsic factor concentrate which is assigned by the N. F. Antianemia Preparations Advisory Board. Vitamin B_{12} with Intrinsic Factor Concentrate conforms to all other requirements under *Antianemia Preparations* [This Interim Revision Announcement, page 5].

Description—Vitamin B_{12} with Intrinsic Factor Concentrate occurs as a dry, slightly yellow, amorphous powder.

Assay for cobalamins—Proceed as directed under *Cobalamin Assay*, page 419. The potency is satisfactory if the result of the assay is not less than 9.0 mcg. and not more than 11.5 mcg. of cobalamins, calculated as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), in each N. F. Unit.

Packaging and storage—Preserve Vitamin B_{12} with Intrinsic Factor Concentrate in tight containers, preferably in a cool place.

Labeling—Label Vitamin B_{12} with Intrinsic Factor Concentrate to show the potency in N. F. Units (oral) assigned to it by the N. F. Antianemia Preparations Advisory Board. The label bears no declaration of the content of vitamin B_{12} activity.

CATEGORY—Hematopoietic.

USUAL DOSE—**1 N. F. Unit** daily.

* * *

Vitamin B_{12} with Intrinsic Factor Concentrate Capsules, page 392

Change the monograph to read:

Vitamin B_{12} with Intrinsic Factor Concentrate Capsules

Vitamin B_{12} with Intrinsic Factor Concentrate Capsules contain the quantity of vitamin B_{12} with intrinsic factor concentrate declared on the label and conform to the requirements under *Antianemia Preparations* [This Interim Revision Announcement, page 5].

Weight variation, page 467—Vitamin B_{12} with Intrinsic Factor Concentrate Capsules meet the requirements of the weight variation test for capsules.

Assay for cobalamins—Proceed as directed under *Cobalamin Assay*, page 419. The potency is satisfactory if the result of the assay is not less than 9.0 mcg. and not more than 11.5 mcg. of cobalamins, calculated as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), in each N. F. Unit.

Packaging and storage—Preserve Vitamin B_{12} with Intrinsic Factor Concentrate Capsules in tight containers, preferably in a cool place.

Labeling—Label Vitamin B_{12} with Intrinsic Factor Concentrate Capsules to show the potency in N. F. Units per capsule assigned by the N. F. Antianemia Preparations Advisory Board. The label bears no declaration of the content of vitamin B_{12} activity.

Capsules available—Vitamin B_{12} with Intrinsic Factor Concentrate Capsules usually available contain the following amounts of vitamin B_{12} with intrinsic factor concentrate: $^1/_2$, $^1/_2$, 1, and 2 N. F. Units.

CATEGORY and DOSE—See Vitamin B_{12} with Intrinsic Factor Concentrate.

* * *

Vitamin B_{12} with Intrinsic Factor Concentrate Tablets, page 393

Change the monograph to read:

Vitamin B_{12} with Intrinsic Factor Concentrate Tablets

Vitamin B_{12} with Intrinsic Factor Concentrate Tablets contain the quantity of vitamin B_{12} with intrinsic factor concentrate declared on the label and conform to the requirements under *Antianemia*

Preparations [This Interim Revision Announcement, page 5].

Disintegration, page 455—The disintegration time limit for Vitamin B_{12} with Intrinsic Factor Concentrate Tablets is 2 hours.

Weight variation, page 468—Vitamin B_{12} with Intrinsic Factor Concentrate Tablets meet the requirements of the weight variation test for tablets.

Assay for cobalamins—Proceed as directed under *Cobalamin Assay*, page 419. The potency is satisfactory if the result of the assay is not less than 9.0 mcg. and not more than 11.5 mcg. of cobalamins, calculated as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), in each N. F. Unit.

Packaging and storage—Preserve Vitamin B_{12} with Intrinsic Factor Concentrate Tablets in tight containers, preferably in a cool place.

Labeling—Label Vitamin B_{12} with Intrinsic Factor Concentrate Tablets to show the potency in N. F. Units per tablet assigned by the N. F. Antianemia Preparations Advisory Board. The label bears no declaration of the content of vitamin B_{12} activity.

Tablets available—Vitamin B_{12} with Intrinsic Factor Concentrate Tablets usually available contain the following amounts of vitamin B_{12} with intrinsic factor concentrate: $^1/_2$, $^1/_3$, 1, and 2 N. F. Units.

CATEGORY and DOSE—See Vitamin B_{12} with Intrinsic Factor Concentrate.

Antianemia Preparations, page 407

Change the entire section to read:

ANTIANEMIA PREPARATIONS

Preparations of liver, stomach, or intestine, individually or in combinations, with or without added vitamin B_{12} activity, that are intended for treatment of pernicious anemia by oral administration require the approval of the N. F. Antianemia Preparations Advisory Board. For Liver Extract, Liver Solution, Liver with Stomach, or Powdered Stomach, 1 N. F. Unit is that amount of an otherwise acceptable product which has been shown to produce, when administered daily by mouth, clinical, and hematopoietic responses in Addisonian pernicious anemia that are considered by the Board to be satisfactory. For Vitamin B_{12} with Intrinsic Factor Concentrate, 1 N. F. Unit is that amount of an otherwise acceptable product which contains an amount of intrinsic factor concentrate shown to produce a satisfactory response in comparison with the response of an N. F. Standard Intrinsic Factor Concentrate when evaluated according to the method *Evaluation of Intrinsic Factor Concentrate.* [This Interim Revision Announcement, page 7.]

A. Applications (5 copies) for approval shall be submitted by manufacturers to the Secretary of the Board.

B. Each application shall include:

(1) Statement of the species of animal tissues used.

(2) Statement of the conditions under which the raw material was obtained, handled, and stored prior to processing. The tissue used in the manufacture of antianemia preparations shall be suitable in quality for use as food by man.

(3) Description of the facilities used in collecting, transporting, storing, manufacturing, and packaging the article.

(4) Description of the methods and the controls used for the manufacture, processing, and packaging of the article. Description and composition of the final product with a statement of the total solids and the amount of the preparation in final form derived from 100 Gm. of original organ. If a product contains material derived from more than one source, such data should be given with respect to each component, except that only the actual amount of vitamin B_{12} activity added, and whether it is in the form of Cyanocobalamin or Cobalamin Concentrate, need be stated.

(5) Description of the controls employed in assuring uniformity in strength, quality, and purity of the drug, and in maintaining the identity of each lot.

(6) a. If the preparation is Liver Extract, Liver Solution, Liver with Stomach, or Powdered Stomach, clinical data obtained by the method *Evaluation of Oral Liver and Stomach Preparations.* [This Interim Revision Announcement, page 6.]

b. If the preparation is Vitamin B_{12} with Intrinsic Factor Concentrate, excretion data obtained by the method *Evaluation of Intrinsic Factor Concentrate.* [This Interim Revision Announcement, page 7.]

(7) All labeling to be used upon the finished product.

C. (1) If the data submitted satisfy the Board that the product as manufactured meets the N. F. requirements, the Board will inform the applicant of the approval of the product and will assign a potency for it, stating the quantity that constitutes 1 N. F Unit.

(2) The potency assigned by the Board shall be stated on the label in terms of the

quantity, in mg., capsule(s), or tablet(s), etc., of material prepared by the method employed in producing the preparation that constitutes 1 N. F. Unit.

Except on containers of material intended for manufacturing use, labels of preparations containing Vitamin B_{12} with Intrinsic Factor Concentrate bear no statement relating amounts of Vitamin B_{12} activity or weight of intrinsic factor concentrate to the assigned potency of the preparations.

The Board will approve abbreviated statements only for small individual containers, in the interest of adequate legibility.

(3) The labeling shall bear a statement of the usual dose of the product. The dose so stated shall be that quantity which provides administration at the rate of not less than 1 N. F. Unit given daily.

Evaluation of Oral Liver and Stomach Preparations

The following procedure is designed for the evaluation of those preparations of the liver or stomach of domestic animals used for food by man which contain factors including Vitamin B_{12} activity which increase the number of red blood corpuscles in the blood of persons affected with pernicious anemia.

Selection of Cases—For the determination of the N. F. Unit potency of liver and stomach derivatives which contain Vitamin B_{12} activity, data shall be submitted to the Advisory Board from observations upon an adequate number of patients (3 cases often should suffice) preferably treated by different lots of material prepared in an identical manner. For such tests, patients must have true Addisonian pernicious anemia with red blood cell counts under 3,000,000 per cubic millimeter. The patients must clearly be in relapse, that is, either must never have received previous effective therapy with liver or stomach preparations, or have received no such therapy within a period of two months prior to the test period. Unless previous experience has shown that a given preparation is reasonably active, no patient in poor clinical condition or whose red blood cell count is dangerously low should be treated with such material except with a large dosage to determine a purely qualitative result. While patients who have no complications, such as chronic infections, marked neurological changes, or arteriosclerosis are preferable for purposes of testing, those showing such changes may be utilized, and in case a positive response is obtained it will naturally furnish valuable evidence. Patients with severe gastrointestinal disturbances should not be used for the testing of preparations for oral use. A brief synopsis of the clinical history, physical examination, and laboratory reports, including gastric analysis, shall be given. Complications and any treatment within the previous two months shall be recorded in the space provided on the second page of the "Confidential Report" form.

Complete "Confidential Reports" shall be submitted on all cases treated with a given dosage of preparation presumed to be an N. F. Unit whether the responses appear to be satisfactory or not.

Hematological Data—While a control period (3 to 5 days) is desirable, a minimum of two consecutive daily observations of the blood, including red cell count, hemoglobin, and reticulocyte percentage, are necessary before the administration of the product to be tested is begun. During the test period a daily reticulocyte count is required until the peak of the rise has been passed and until for three consecutive days the reticulocyte percentage has returned approximately to the pre-treatment level. The reticulocytes should be counted in fixed dry preparations previously stained with brilliant cresyl blue or new methylene blue. The blood films should be filed as a permanent part of the record so that they may be examined in case of question as to the nature or the degree of the response. Data covering the red blood cell count and hemoglobin or volume of packed red cells are necessary at least twice a week during the first 3 weeks of treatment, and if possible, thereafter at least once a week until the blood has returned to normal. Data concerning the red blood cells, hemoglobin or hematocrit, and reticulocytes are to be recorded on the third page of the "Confidential Report" form. The red blood cell values are to be plotted on the final page of the form. In the column headed "Notes" shall be recorded any evidence of undesirable effects such as nausea, vomiting, or diarrhea. Beneficial effects, such as abolition of glossitis, diarrhea, or paresthesias, also shall be recorded.

Diet—During at least the control period and the reticulocyte response the patient should omit meat, eggs, and whole grain cereals from the diet. Experience has shown that occasionally such substances interfere with the test and that their omission for a short time is not harmful to the patient. Except for the preparation under test, no liver, kidney, stomach, or yeast, or any derivative of these, or any special medication containing vitamins, arsenic, or iron, should be given during the period of observation.

Definition of N. F. Unit—The clinical data submitted shall satisfy the Board that the daily (oral) administration of a given uniform amount of the material in question has produced, under adequately controlled conditions, a satisfactory clinical and hematopoietic response in pernicious anemia. Table I gives values for reticulocyte re-

sponses. Curves of the typical response in the red blood cell count to effective therapy are familiar to hematologists generally, and such a curve should be submitted for each patient, on the "Confidential Report" form.

Table I—Average "Peak" Reticulocyte Responses for Various Initial Red Blood Cell Counts

Initial Red Blood Cell Count (Millons per Cubic Millimeter)	Peak of Reticulocyte Curve (Per Cent)
1.0	41.8
1.5	28.4
2.0	18.6
2.5	11.1
3.0	5.1

Because of the recognized difficulties of securing numerous tests, the particular daily dosage of the product which, in the opinion of the Board, has produced a satisfactory clinical and hematopoietic response will be considered as an N. F. Unit unless, as a result of further observations, smaller amounts of material are approved by the Board as constituting an N. F. Unit. It is thus apparent that the designation of 1 N. F. Unit indicates only the particular amount of material which has been approved and not necessarily the minimum amount of the particular product which might be so approved by further tests with small dosage. N. F. Units of different preparations are therefore not necessarily strictly comparable in potency.

Selection and Administration of Presumptive N. F. Unit Dosage—Preliminary observations on one or more patients with Addisonian pernicious anemia are necessary in order to determine that the preparation is without undesirable effects and in order to give a rough idea of its therapeutic effectiveness. For the purpose of establishing the N. F. "Unit" potency of preparations, it is then necessary to choose a presumptively sufficient amount of the preparation and to administer it daily in the uniform dosage chosen. The daily oral administration must be continued for at least 3 weeks and preferably until the blood values have returned to normal. Great care should be exercised in measuring and in administering exactly the specified daily dosage.

Evaluation of Intrinsic Factor Concentrate

The following procedure is designed for the evaluation of those preparations of the stomach or intestine of domestic animals used for food by man which when combined with Vitamin B_{12} activity will increase the absorption of the latter from the gastrointestinal tract of patients suffering from pernicious anemia. The handling of radioactive preparations and the counting techniques should be conducted by personnel having had expert training and experience in such techniques.

[NOTE—An interval of not less than 72 hours must elapse between each oral administration of isotope-labeled cyanocobalamin.]

Standardization of patients—Select pernicious anemia patients in a satisfactory physiological state. The patient is satisfactory if the *Baseline response* is not more than 3 per cent. It is preferable that the *Baseline response* be less than 2 per cent. Restandardize each patient after obtainiag five consecutive *Sample responses* or if more than 4 weeks have elapsed since obtaining the preceding *Sample response*.

A. *Baseline response*—Fast the patient for a minimum of 4 hours. Administer orally to the patient 2 mcg. of cyanocobalamin which has been labeled with a suitable radioisotope of cobalt. Adjust the specific activity of the cyanocobalamin so that the amount of the radioactivity which is excreted in the urine is sufficient for obtaining suitable accuracy. Immediately following the administration of the oral dose, administer intramuscularly to the patient a flushing dose of 1,000 mcg. of nonlabeled cyanocobalamin. Collect and pool all the urine excreted by the patient during the following 24-hour period. At the end of this period administer intramuscularly a second flushing dose of 1,000 mcg. of nonlabeled cyanocobalamin. Determine, by suitable means, the total radioactivity excreted in the 24-hour urine collection and express as a percentage of the total orally administered radioactivity. This is designated as the *Baseline response*.

B. *Standard response*—Proceed as described under *Baseline response*, except administer simultaneously with the 2 mcg. oral dose of radioisotope-labeled cyanocobalamin 1 N. F. Unit of N. F. Reference Standard Intrinsic Factor Concentrate. Express the total radioactivity excreted in the 24-hour urine collection as a percentage of the total orally administered radioactivity. This is designated as the *Standard response*.

Evaluation of preparations—Select the preparation to be used for evaluation from a representative production batch prepared by the method described in the application.

Proceed as described under *Baseline response*, except administer simultaneously with the 2 mcg. oral dose of isotope-labeled cyanocobalamin an amount of the preparation to be evaluated which is expected to be equivalent to 1 N. F. Unit. Express the

amount of the total radioactivity excreted in the 24-hour urine collection as a percentage of the total orally administered radioactivity. This is designated as the *Sample response*.

Using *Standard responses* and *Sample responses* obtained from not less than three patients, calculate the average *Standard response* and the average *Sample response*. A preparation is considered satisfactory if the average *Sample response* is not less than 80 per cent of the average *Standard response*.

Containers—Glass, page 421

In the first sentence of the paragraph entitled *Special Distilled Water*, change . . .between 0.5 and 1 mho to read:

. . .between 0.5 and 1 μmho.

In the table, *Glass Types and Test Limits*, page 422, in the left-hand column under *Limits* for Type II (Treated soda-lime glass), change *All* to read:

Over 160.

In the left-hand column under *Limits* for Type III (Soda-lime glass), change *15 or less* to read:

All.

Glass Types, page 422

Change the last sentence of the paragraph to read:

Type III containers should be sterilized by dry heat prior to filling.

Light Transmission, page 422

Postpone the effectiveness of the entire section on *Light Transmission* [beginning on page 422] until further notice.

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should include a summary to be used as an introductory synopsis of not more than 150 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

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(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

References to books should be in the order given (1) author's surname and initials, (2) title of the book, (3) volume, (4) edition, (5) name of publisher, (6) place of publication, (7) year of publication, and (8) page number. A complete book reference may be illustrated as follows:

(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages, and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e. g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mcg., mm., cm., and L.

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Solubilization of Riboflavin by Complex Formation with Caffeine, Theophylline, and Dimethyluracil*

By DAVID E. GUTTMAN and MANOHAR Y. ATHALYE†

The demonstration of complex formation between riboflavin and caffeine by previous workers suggested that xanthines and related compounds might be suitable as solubilizing agents for the vitamin. Caffeine, theophylline, and dimethyluracil were investigated and were found to increase the apparent water solubility of riboflavin. The effect was presumably due to the formation of 1:1 complexes. Apparent dissociation constants were determined to be: caffeine, 34.5×10^{-3} mole/L.; theophylline, 52.6×10^{-3} mole/L.; dimethyluracil, 182×10^{-3} mole/L. The marked difference in complexing tendencies between caffeine and dimethyluracil suggested that the imidazole ring of the xanthine nucleus was strongly involved in the interaction. Quenching of fluorescence experiments were also conducted for comparative purposes.

RIBOFLAVIN is a component of many types of multivitamin dietary supplements including vitamin tablets, capsules, oral fluids, and parenterals. Prior to the availability of water-soluble salts of riboflavin-5′-phosphate, the incorporation of the vitamin into the two latter classes of dosage forms was a difficult problem for the pharmaceutical formulator due to its limited solubility in water. In many cases this difficulty was overcome by incorporating an auxiliary agent into the vehicle which had a specific solubility-enhancing effect on riboflavin. The use of these so-called solubilizers has been a common practice and many have been reported in both the scientific and patent literature. Some of the many agents which have been suggested for this purpose are listed in Table I.

TABLE I.—SUGGESTED SOLUBILIZERS FOR RIBOFLAVIN

Agent	Reference
Nicotinamide	(1)
2,4-Dihydroxybenzoic acid and its monoalkyl ether	(2)
Acetamidine HCl, Na *p*-aminobenzoate, monoethanolamine salicylate, Na *p*-hydroxybenzoate	(3)
Sodium benzoate, sodium salicylate	(4)
Sodium saccharin	(5)
Gallic acid	(6)
Vanillin	(7)
Veratryl alcohol	(8)
Tyrosine amide	(9)
Salts of hydroxybenzoic acid	(10)
Tryptophan	(11)
3-Pyridylcarbinol	(12)
Na-3-hydroxynaphthoate	(13)
Urea and urethane	(14)
3-Hydroxy-7-sulfo-2-naphthoic acid	(15)
Na desoxycholate, N-methylacetamide	(16)

Although most of these agents were discovered as a result of empirical investigations, limited evidence indicates that the mechanism of solubilization in a number of cases involved complex formation between riboflavin and the solubilizing agent. Frost (1), for example, investigated the effect of nicotinamide, a widely used solubilizer, on the solubility of riboflavin in water. He found that at pH 5, the solubility increased from approximately 0.1 to 2.5 per cent with an increase in nicotinamide concentration from 5 to 50 per cent. At pH values below 5, a

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decrease in this solvent effect was apparent. The results suggested that the solubility-enhancing effect was due in part to salt formation and in part to complex formation.

The interaction of riboflavin with phenol in aqueous solution was studied by Yagi and Matsuoka (17) by both spectral and quenching of fluorescence studies. The near ultraviolet and visible absorption spectrum of riboflavin was found to shift in the direction of longer wavelengths in the presence of phenol. The results were interpreted on the basis of 1:1 complex formation and a dissociation constant of 0.21 mole/L. at 20° was calculated. Complex-formation between the interactants was also suggested by the marked inhibition of the fluorescence of riboflavin in aqueous solution caused by the addition of phenol. Quenching of fluorescence experiments yielded a dissociation constant which was, however, approximately one-ninth that derived from spectral studies. The authors felt that the marked quenching of fluorescence could not be explained solely on the basis of the formation of a nonfluorescent interaction product but must involve other factors. It will be seen that a similar situation was encountered in this investigation.

The effects of various substances on the water-solubility and other properties of riboflavin were investigated by Sakai (18). Compounds such as resorcinol, pyrogallol, hydroquinone, phloroglucinol, *p*-aminobenzoic acid, anthranilic acid, phthalic acid, benzoic acid, and others were found to exhibit solubilizing action. Related aliphatic compounds did not. Hydrogen-bond formation was postulated between the vitamin and some of the solubilizers. Harbury and Foley (19), however, studied the interactions of various isoalloxazine derivatives and conjugated molecules by spectral measurements and determined that in several systems where no possibility of hydrogen-bond formation existed an interaction did, in fact, occur. They suggested that hydrogen-bonding is not a primary feature of such interactions but that molecular charge transfer and other mechanisms may be more important. The results of the present investigation support this view.

The objective of this investigation was to study caffeine and some related compounds as potential solubilizers of riboflavin. It was expected, on the basis of structural similarities between riboflavin and other compounds which have been shown to complex with caffeine, that interactions and subsequent solubilization would occur. A search of the literature revealed that complex formation between caffeine and riboflavin and between caffeine and 3-methylriboflavin was indeed known. Quenching of fluorescence studies and spectral studies were utilized by Weber (20) and Yagi and Matsuoka (17), respectively, to determine the dissociation constant of a 1:1 complex which formed between caffeine and riboflavin. The value of 0.019 mole/L. at 20° determined by the latter workers did not agree with the value of 0.011 mole/L. obtained by Weber at approximately the same temperature. Harbury and Foley (19) studied the caffeine-3-methylriboflavin system and determined by spectral studies a dissociation constant of 0.021 mole/L. at 22.5°. These constants must be considered as approximate since the nonideal nature of caffeine in aqueous solution was not recognized by the investigators.

Of pharmaceutical interest is the application of these interactions to increase the apparent solubility of riboflavin in a dosage form. Although previous workers were not motivated by this objective, their results recommend this extension. For example, the dissociation constant for the riboflavin-caffeine complex as given by Yagi and Matsuoka suggests that the solubility of riboflavin in a 2 per cent solution of caffeine would be approximately six times that in water alone. The present investigation was designed to test the anticipated riboflavin-solubilizing ability of caffeine, theophylline, and dimethyluracil and to gain additional information concerning the nature of the interactions.

RESULTS

Solubility Studies.—Solubilization through complex formation is attributed to the formation of a new species in the solution phase which has a solubility, independent of that of the parent compound. In such a system, two simultaneous equilibria are operative. One involves the reversible transfer of solute from the solid phase to the solution phase while the other is the reversible association of interactants to form the complex. From this consideration it can be shown that in a system where a complex of 1:1 stoichiometry is formed, solubility relationships can be described by

$$S/S_0 = 1 + (C/K) \qquad \text{(Eq. 1)}$$

where S_0 = solubility of solute in the absence of complexing agent, S = apparent solubility of solute in the presence of the complexing agent, C = molar concentration of complexing agent, and K = dissociation constant of the complex.

Equation 1 assumes that activities of the species can be represented by concentration terms and that the activity of the solvent is not markedly affected by the presence of complex and/or complexing agent. If the stoichiometric concentration of complexing agent is large compared to the concentra-

tion of complex, then, Eq. 1 can be approximated by

$$S/S_0 = 1 + (C_t/K) \qquad \text{(Eq. 2)}$$

where C_t = stoichiometric concentration of complexing agent.

Equation 2 thus provides a graphical method for the determination of the dissociation constant.

Results of the solubility studies are shown in Fig. 1 where the function S/S_0 is plotted as a function of the concentration of complexing agent. Definite interactions are indicated by the results. It can be seen, for example, that the solubility of riboflavin in a 0.0927 M solution of caffeine in water exceeded that in water alone by a factor of three. No difference in solubilizing action was found when 0.087 M acetic acid was used in place of distilled water. This suggests that the interaction was independent of pH, at least in the acid to neutral range.

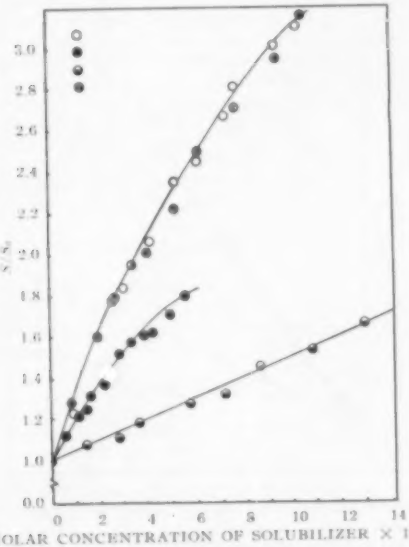


Fig. 1.—The solubility of riboflavin in solutions of caffeine, theophylline, and dimethyluracil at 30°. ○, Caffeine,; ●, theophylline; ◒, dimethyluracil; ⊗, Caffeine at pH 4.2.

Marked deviations from the expected linearity of this plot are apparent in the case of caffeine and theophylline. The deviations are not unexpected in view of the reported nonideal behavior of caffeine and theophylline in aqueous solution (21). The deviations are such that they can be explained on this basis. In dilute solutions where the xanthines existed in essentially monomeric form, deviations from linearity were small. At higher concentrations, where dimeric and tetrameric species predominate, the deviations were correspondingly marked. It was interesting to observe that linearity throughout a wide range of concentration held for dimethyluracil. This behavior strongly suggests that in contrast to caffeine and theophylline, dimethyluracil does not undergo marked self-complexing in aqueous solution. Because of this, dimethyluracil might be a desirable agent for basic studies of caffeine-type complex formation.

Dissociation constants were calculated from the slopes of the curves of Fig. 1. The initial straight line portions of the curves representing caffeine and theophylline were used for this purpose. The values obtained must be assumed to represent apparent dissociation constants which are valid only for comparative purposes. The constants are tabulated in Table II. It is seen that the tendency for complex formation is greatest with caffeine and significantly smaller with dimethyluracil. The constant calculated for the caffeine-riboflavin complex is not in agreement with values determined by previous workers, especially that of Weber. The degree of complex formation indicated by Weber's study was much greater than that determined in the present investigation. This discrepancy motivated a further study, essentially an extension of Weber's work, in attempt to explain the lack of agreement between experimental methods.

TABLE II.—DISSOCIATION CONSTANTS OF SOME COMPLEXES OF RIBOFLAVIN AT 30° ± 0.5°

Complexing Agent	Dissociation Constant, Mole/L.
Caffeine	34.5×10^{-2}
Theophylline	52.6×10^{-2}
Dimethyluracil	$182. \times 10^{-2}$

Quenching of Fluorescence Studies.—A common observation is quenching of fluorescence, a reduction in or an elimination of the fluorescence of compounds, caused by changes in temperature, solvent, physical state, or by the presence of quenching agents. The mechanism of action of the latter can be quite complex. The quenching effect can be due to collisions between a quencher and the fluorescent species, or to a nonspecific transient interaction between them, or a combination of the two effects. Alternatively, apparent quenching can result from a strong interaction which results in the formation of a nonfluorescent complex.

The marked quenching effect exhibited by caffeine on the fluorescence of riboflavin in aqueous solutions is illustrated in Fig. 2. Here the intensity of fluorescence of a solution containing a riboflavin concentration of 1 mcg./ml. is plotted *versus* the concentration of caffeine in the solution. It can be seen that at a caffeine concentration of 0.1 mole/L., for example, an approximate 90% decrease in fluorescence occurred. Similar results were obtained at riboflavin concentrations of 0.50 and 0.25 mcg./ml.

If the observed quenching effect was due solely to the formation of a nonfluorescent complex of 1:1 stoichiometry, then, according to the treatment of Weber, it can be shown that

$$I_0/I = I + (\text{Caffeine}/K) \qquad \text{(Eq. 3)}$$

where I_0 = intensity of fluorescence in the absence of caffeine, I = intensity of fluorescence in the presence of caffeine, caffeine = molar concentration of caffeine, and K = dissociation constant of the complex.

If the concentration of caffeine is large compared

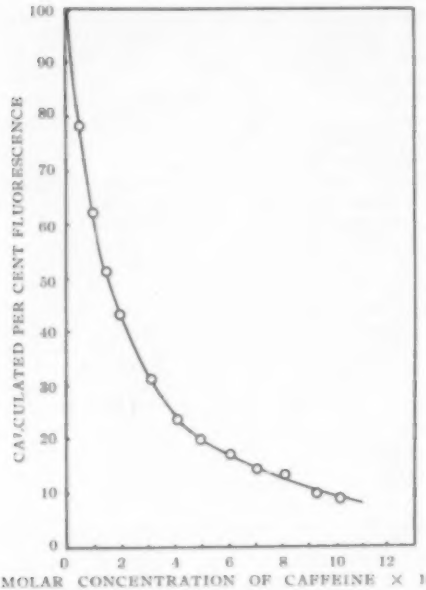


Fig. 2.—The quenching of riboflavin fluorescence by caffeine. Riboflavin concentration = 1 mcg./ml.

to that of the complex, Eq. 3 can be approximated by

$$I_0/I = 1 + (\text{Caffeine}_t/K) \qquad \text{(Eq. 4)}$$

where Caffeine$_t$=stoichiometric concentration of caffeine.

A plot of I_0/I *versus* (caffeine)$_t$ provides the means to test the assumptions and to determine the dissociation constant graphically. The results with caffeine at three different concentrations of riboflavin are plotted in this manner in Fig. 3. The lack of linearity of the experimental curves and the different curves obtained at the different concentrations of riboflavin indicate that quenching is not entirely due to the formation of a nonfluorescent complex. The upward curvature of the lines strongly suggests that other quenching mechanisms were also operative. The dissociation constant of 14.6×10^{-3} mole/L. calculated from the initial linear portion of the curves approximates that determined by Weber. This agreement indicates the reproducibility of the method but does not substantiate the validity of the basic premises. The quenching effects of theophylline and dimethyluracil were also investigated and the results are summarized in Fig. 4 where a caffeine curve was also plotted for comparative purposes. It will be noticed that this method failed to distinguish between the complexing tendencies of caffeine and theophylline. A definite difference was apparent from solubility experiments. Although the curve for dimethyluracil is linear, the dissociation constant calculated from this plot is only one-fifth that obtained from solubility experiments. In all cases the fluorescence studies indicated a much greater degree of complex formation than corresponding solubility studies. Complexing of riboflavin by these agents undoubtedly contributes in part to the overall degree of quenching, however, additional mechanisms must also be operative. Similar observations were made by Yagi and Matsuoka (17) on the phenol-riboflavin system.

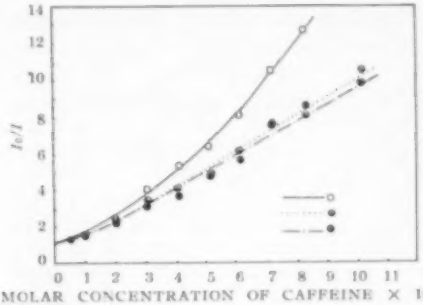


Fig. 3.—The quenching of riboflavin fluorescence by caffeine. Riboflavin concentration: ○, 0.25 mcg./ml., ◒, 0.50 mcg./ml.; ●, 1.00 mcg./ml.

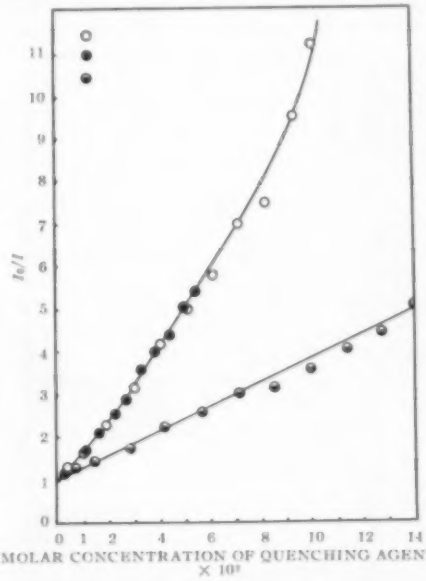
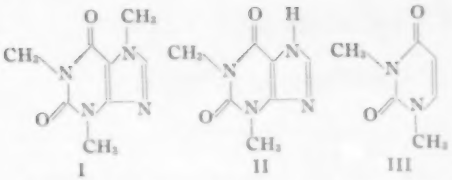


Fig. 4.—The quenching of riboflavin fluorescence by caffeine, theophylline, and dimethyluracil. Riboflavin concentration = 1 mcg./ml. ○, Caffeine; ●, theophylline; ◒, dimethyluracil.

DISCUSSION

Some insight into the nature of the interaction between riboflavin and the three compounds studied can be obtained by comparing complexing tendencies. The stabilities of the complexes formed with caffeine (I) and theophylline (II) were found to be many times greater than that of dimethyluracil (III).



I II III

The imidazole ring of the xanthine nucleus must then be strongly involved in the interaction. This supports the suggestion of Harbury and Foley (19) that hydrogen bonding may not be the main interaction component of isoalloxazine-xanthine complexes. The three compounds studied in this investigation all contain the 1,3-dimethylpyrimidinedione nucleus, a potential center for hydrogen-bond formation. Nevertheless, the stability of the caffeine complex is approximately five times that of the dimethyluracil product. It is possible, however, that hydrogen bonding provides a primary linkage between the interacting molecules and is reinforced by secondary van der Waal interactions which involve other parts of the molecule. Interpretations are equivocal however, in view of the limited data available.

It is interesting to compare caffeine with other compounds reported in the literature as solubilizers of riboflavin. The solubility of riboflavin in 0.1 *M* solutions of nicotinamide (1), vanillin (7), gallic acid (6), and acetamidine HCl (3) were interpolated from the data presented in the references to be 0.66×10^{-3}, 0.60×10^{-3}, 0.69×10^{-3}, and 0.25×10^{-3} mole/L., respectively. In comparison, the solubility in a solution of caffeine of corresponding concentration was 1.27×10^{-3} mole/L. It seems quite probable that structural modifications of the xanthine nucleus might yield compounds capable of more pronounced solubilizing action by virtue of stronger complexing tendencies.

EXPERIMENTAL

Materials.—Riboflavin was obtained from Eastman. Caffeine and theophylline were U. S. P. grade. Dimethyluracil was synthesized according to the procedure of Davidson and Baudisch (22).

Solubility Studies.—Ten-milligram quantities of riboflavin were weighed into a series of 25-ml. glass-stoppered Erlenmeyer flasks. Distilled water and/or a solution of the agent under investigation was added by buret to each flask to make a total volume of 20 ml. The flasks were stoppered, placed in a constant temperature bath at 30±0.5°, and agitated for sixteen hours. Agitation was stopped and the undissolved powder was permitted to settle. An aliquot of each solution was removed and rapidly filtered with the aid of a Swinney filter adaptor attached to a syringe. One milliliter of the filtrate was diluted with distilled water to 500 ml. in a volumetric flask. Approximately 50 ml. of the solution was extracted three times, each with 20 ml. of chloroform. Preliminary experiments showed that this method effected the desired extraction of caffeine and that riboflavin was not extracted from its aqueous solution. The aqueous solution of riboflavin was then gently warmed on a water bath to remove any trace of chloroform. This precaution was necessary since chloroform was found to exert a slight quenching effect. The concentration of riboflavin was then determined fluorimetrically with a Lumetron fluorimeter. The instrument was adjusted to 100% intensity with a 1.5 mcg./ml. solution of riboflavin. A standard curve was obtained by using known concentrations of the vitamin. This curve was used to determine the riboflavin content of all solutions.

Quenching of Fluorescence Studies.—A solution containing 50 mcg./ml. of riboflavin was prepared according to the directions of the "United States Pharmacopeia XV" (23). An aliquot of this was transferred to each of a series of 100-ml. volumetric flasks. Distilled water and/or a solution of the agent under investigation was added by buret to each flask to make a total volume of 100 ml. The fluorescent intensity of each solution was then determined with a Lumetron fluorimeter.

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Antibacterial Activity of Essential Oil Vapors*

By JASPER C. MARUZZELLA and NICHOLAS A. SICURELLA

Vapors of 133 essential oils were screened *in vitro* for antibacterial activity against six test organisms. One hundred and five vapors were found to possess antibacterial activity on at least one of the test organisms. Vapors of thyme (white), cassia, thyme (red), savory, cinnamon, origanum, and cherry laurel produced prominent zones of inhibition with all test organisms. Gram-positive bacteria were more susceptible to the vapors than Gram-negative bacteria.

STUDIES on the action of essential oil vapors on anthrax spores were reported in 1887 by Chamberlain (1). Subsequent investigations by Greig-Smith (2), Macht and Kunkel (3), Morel and Rochaix (4, 5), Schobl and Kusama (6), Coulthard (7), Remlinger and Bailly (8), and recently by Ryu (9) and Grubb (10) have clearly demonstrated that essential oil vapors possess antibacterial properties. Yet the proper evaluation of these volatile plant products on bacteria cannot be fully realized because of the diverse methods used by various investigators and the relatively small number of essential oil vapors tested. This study was undertaken in an attempt to evaluate a large number of essential oil vapors against a variety of Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Vapors of 133 essential oils were screened for antibacterial activity against the following test organisms: *Escherichia coli* (B strain), *Staphylococcus aureus* (ATCC 10390), *Bacillus subtilis* var. *aterrimus* (ATCC 6461), *Streptococcus fecalis*, *Salmonella typhosa* (ATCC 10794), and *Mycobacterium avium* (ATCC 4676). Twenty-four-hour cultures of these organisms were grown in nutrient agar and broth (Difco) at 37° except for *M. avium* which was grown for seventy-two hours in 5% glycerol broth and agar.

The detection of essential oil vapors for antibacterial activity was conducted in the following manner. Approximately 18 cc. of melted agar were poured into Petri dishes and allowed to solidify. To the surface of the agar was added 0.2 cc. of bacterial broth culture which was spread with a glass spreader with the aid of a turntable. Filter paper disks (6.35 mm. diameter) were completely saturated with the oil to be tested and placed in the center of the inner surface of the Petri dish cover. Thus, when the dishes were inverted and incubated, the saturated disks were at a distance of about 8 mm. from the surface growth of the organism. All dishes were incubated at 37° for twenty-four hours except those with *M. avium*, which were incubated for seventy-two hours. The presence of a clear zone on the surface of the agar above the disk was indicative of antibacterial activity, and degree of activity was determined by the size of the zone of inhibition. The diameter of the zones of inhibition was measured to the nearest millimeter by means of a metric ruler with the aid of an illuminated Quebec colony counter. All plates were conducted in quadruplicate with one disk per dish. Each measurement therefore represents a mean value of four recordings. While it is recognized that day-to-day fluctuations in the resistance of the test organisms may have occurred, they are not considered to affect the overall screening of the vapors against the bacteria appreciably.

RESULTS AND DISCUSSION

Vapors of 133 essential oils were used in conducting 784 tests on the six test organisms. In this series of tests, the vapor showed antibacterial activity in 179 (23%) cases. Table I lists the essential oil vapors together with their zones of inhibition. Of the 133 oil vapors tested, 71% were found to be active against *M. avium*, 19% against *B. subtilis* var. *aterrimus*, 12% against *S. fecalis*, and 6% against *E. coli*. Of the one hundred and nineteen oil vapors screened against *S. aureus*, 14% showed activity. Thus, *M. avium* was the most susceptible organism while *E. coli* was the most resistant. Moreover, the Gram-positive bacteria were more vulnerable to essential oil vapors than the Gram-negative.

Further inspection of the data reveals that the vapors of the following oils produced the largest zones of inhibition on all test organisms: thyme

TABLE I.—INHIBITORY ACTIVITY OF ESSENTIAL OIL VAPORS

Oils[a]	Diameter of Zones of Inhibition, mm.[b] *S. aureus*	*B. subtilis* var. *aterrimus*	*S. fecalis*	*S. typhosa*	*M. avium*
Abies alba (from needles)	0	0[c]	0	0	90
Abies Sibirica	0	0	0	0	67
Amyris	0	0	0	0	46
Angelica root	0	0	0	0	51
Angelica seed	0	0	0	0	29
Anise, U. S. P.	0	0	0	0	54
Basil, sweet	0	0	0	0	32
Bay N. F.	0	0	0	0	44
Bergamot N. F.	0	0	0	0	52
Birch tar, rectified	0	0	0	0	36
Bois de rose	0	6	0	19	90
Cade, rectified U. S. P.	0	0	0	0	64
Cajuput	0	0	0	0	62

* Received February 2, 1960, from the Department of Biology, Long Island University, Brooklyn, N. Y.

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TABLE I.—(*Continued*)

Oils[a]	Diameter of Zones of Inhibition, mm.[b]				
	S. aureus	*B. subtilis* var. *alerr-imus*	*S. fecalis*	*S. typhosa*	*M. avium*
Cananga, rectified	0	0	0	0	60
Caraway	..[d]	0	0	0	60
Cardamom	0	0	0	0	53
Cascarilla	0	0	0	0	32
Cassia U. S. P.	25	22	44	19	60
Cedar leaf	..	23	0	0	70
Cedar wood	0	29	0	0	68
Celery seed	0	0	0	0	20
Chenopodium N. F.	0	0	0	0	69
Cherry laurel	..	0	90	90	90
Cinnamon, Ceylon	28	20	31	24	54
Citronella, Formosan	0	54	0	0	90
Clove U. S. P.	0	0	0	22	43
Clove leaf, rectified	0	0	0	0	31
Cognac green	0	0	0	0	64
Copaiba	0	0	0	0	30
Coriander U. S. P.	0	9	0	21	70
Cubeb	0	15	0	0	60
Cumin	..	6	0	0	70
Curacao peel	0	0	0	0	16
Cypress	0	0	0	0	50
Dill seed	0	0	0	0	90
Dill weed	..	0	0	0	67
Erigeron	0	0	0	0	22
Estragon	0	0	0	0	25
Eucalyptus, rectified N. F.	0	0	17	0	62
Fennel U. S. P.	0	0	0	0	68
Galbanum	..	0	0	0	23
Garlic, imported	..[e]	24	0	0	90
Geranium, Algerian	0	0	0	0	64
Ginger	0	0	0	0	22
Gingergrass, native	0	0	0	0	57
Hemlock	0	0	0	0	64
Labdanum	..	0	0	0	22
Laurel leaf	0	0	0	0	29
Lavandin	0	0	0	0	90
Lavender U. S. P.	0	0	0	0	82
Lemongrass, rectified	33	42	29	0	90
Linaloe wood	0	0	0	12	73
Mace	0	0	0	0	44
Majoram, sweet	0	11	0	0	68
Melissa balm (so-called)	0	0	0	0	67
Mountain laurel	0	0	0	0	36
Myrtle (so-called)	0	0	0	0	62
Neroli, bigarade petale, N. F.	0	0	0	0	66
Nutmeg, East Indian U. S. P.	0	0	0	0	62
Nutmeg West Indian U. S. P.	0	0	0	0	24
Ocotea Cymbarum	0	0	0	0	27
Olibanum	..	0	0	0	33
Onion	..	26	0	0	90
Orange, bitter	0	0	0	0	21
Origanum	18	20	17	19	39
Origanum, rectified water white	18	22	20	22	49
Palmarosa	0	0	0	0	46
Patchouly, Singapore	0	28	0	0	60
Pennyroyal, imported	..	0	0	0	65
Pepper, black	0	0	0	0	46
Peppermint, natural	8	17	0	0	90
Peppermint, rectified U. S. P.	10	0	0	0	63
Petitgrain, Paraguay	0	0	0	0	65
Pimenta (from berries)	0	0	0	22	43
Pimenta leaf	0	0	0	29	38
Rhodium	0	0	0	7	71
Rose, kazanlik, U. S. P.	0	0	0	0	43
Rosemary, N. F.	..	0	0	0	25
Rosemary, acetylated	..	0	0	0	63
Rue	0	0	0	0	90
Rusci, rectified	0	0	0	0	56
Sage, Dalmatian	..	16	0	0	69
Sage, Clary	0	0	0	0	62
Sandalwood	0	0	0	0	25
Sassafras N. F.	0	0	0	0	37
Savin	0	0	0	0	72
Savory select	34	24	17	17	68
Snakeroot, Canada	0	0	0	0	27
Spearmint, N. F.	..	0	0	0	54
Spike lavender	15	0	0	0	74
Spike lavender, acetylated	0	0	0	0	90
Spruce	0	18	0	0	90
Sweet birch, northern U. S. P.	0	0	0	0	90
Tansy	..	0	0	0	72
Tar, rectified, N. F.	0	0	0	0	68
Thyme, red N. F.	25	28	25	25	57
Thyme, white N. F.	26	24	20	27	51
Ti-Tree, Australian	0	0	0	0	69
Turpentine, rectified N. F.	10	0	21	18	60
Valerian, Indian	10	10	7	0	34
Verbena	10	17	0	0	90
Vetiver, Haiti	0	0	0	0	14
Wintergreen, northern U. S. P.	0	0	0	0	90
Wormwood	..	0	0	0	66
Ylang Ylang	0	0	0	0	29

[a] The essential oils were generously supplied by Magnus, Mabee, and Reynard, Inc., New York, and Fritzsche Brothers, Inc., New York.

[b] The following essential oil vapors were active against *E. coli*: cassia U. S. P. (20 mm.), cherry laurel (90 mm.), cinnamon (Ceylon) (19 mm.), eucalyptus, rectified N. F. (15 mm.), origanum (21 mm.), savory, select (20 mm.), thyme red N. F. (28 mm.), and thyme white N. F. (29 mm.).

[c] Zone of inhibition absent.

[d] Not tested.

[e] Reduced growth as compared to control with no definite zone of inhibition.

(white), cassia, thyme (red), savory, cinnamon (Ceylon), origanum, and cherry laurel.

The 28 oil vapors with no activity were: *Abies alba* (from cones); amber, rectified; balsam Peru; calamus; camphor, sassafras; camphor, white; chamomile, German; dwarf pine needle; grapefruit, Florida expressed; guaiac wood; hops; juniper, twice rectified; lemon, California coldpressed U. S. P.; lemon, Italian handpressed U. S. P.; lime, distilled; lime, expressed; lovage; mandarin, Italian; myrrh; niaouli; opopanax; orange, California sweet, coldpressed U. S. P.; orris root, Florentine; parsely seed; persic U. S. P.; *Pinus sylvestris;* styrax; and tangerine.

Why *M. avium* should be so susceptible to essential oil vapors is not known. One possible explanation might be the fact that this organism was exposed to the vapors for a longer period of time (seventy-two hour incubation period) and hence greater susceptibility due to greater absorption of the vapors on to the agar. One might speculate as to the possible mode of action involving a penetration of the essential oil vapor into the cell barrier disrupting the chemical organization (lipids) of the cell.

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Growth and Differentiation of *Atropa belladonna* L. as Affected by Different Sources of Nitrogen*

By L. J. SCHERMEISTER†, F. A. CRANE, and R. F. VOIGT

Growth and morphology were compared on plants furnished six levels of nitrate and three of ammonia in water culture. The production of dry weight with ammonia was significantly greater than with nitrate. Ratios of shoot/root and leaf/stem indicated that use of the nitrogen was very different at low vs. high levels, particularly with ammonia nitrogen.

MANY LITERATURE reports relating the use of fertilizer combinations to alkaloid production in the solanaceous drug plants have led to a vague understanding of what the plant needs and how it is used. In this study several different nitrogenous sources were furnished belladonna plants grown in nutrient solutions and the patterns of growth and maturation were observed and described.

Growth studies reported on the belladonna plant indicate that a nitrogen source furnished early enough in the vegetative growth period significantly increases plant production and stimulates alkaloid synthesis (1–7). They did not make clear the advantages of one form of nitrogen over another nor did they deal with the problem of availability of the nutrients to the plant from the various types of manures or soils in which the plants were grown. The reference of James (4) to the improper balance of fertilizer elements further points up the need for growing the plants in a controlled medium in which purified chemicals are the only nutrients available. Since the belladonna plant is capable of synthesizing its nitrogenous constituents from inorganic sources, plants were grown in nutrient solutions containing several concentrations of nitrate and ammonium salts. Plants were grown through a standard growth period to a definite flowering stage; were harvested, and representative samples were prepared for the various determinations reported later. During the growth periods measurements were made that characterize the features of this plant as they were affected by nutrition. Ratios of various plant parts to each other indicate the effect of nutritional change on morphology.

EXPERIMENTAL PROCEDURE

Plants used in all experiments were germinated from good quality acid-scarified seed in a sand-vermiculite mixture maintained at 70° F. Germination occurred within two to three weeks.

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Following germination, the young seedlings were transferred to a full nutrient solution (8) where they were prepared for the experimental treatments.

Plants were grown in 3-gallon glazed crocks, supported on specially designed masonite covers. They were lightly fastened by plant ties to the wire frame as their size required.

Nutrient solutions were prepared from reagent grade chemicals and distilled water on a mg. equiv. per L. basis. The composition of nutrient solutions is shown in Table I. The levels of nitrogen were varied both above and below the level of the well-known Hoagland and Arnon solution (15 mg. equiv./L.) which was considered a standard or control concentration. A preliminary experiment had shown that belladonna plants could not tolerate ammonia as a sole nitrogen source in culture solution. Therefore, ammonia at three levels was supplemented by nitrate in a 1–4 ratio as shown in Table I. Distilled water was added periodically to maintain volumes and the pH of solutions was adjusted to 5.8 weekly with dilute hydrochloric acid.

TABLE I.—COMPOSITION OF NUTRIENT SOLUTIONS USED FOR GROWING *Atropa belladonna* L.[a]

All solutions contained the following ions mg. equiv./L.			
Calcium	10	Phosphorus	1
Potassium	4	Sulfur	4
Magnesium	4	Trace elements (H and A mixture)	1 per week
Iron (as citrate)	1 per week		
Experimental solutions contained in addition to those above			
Nitrogen as Nitrate	5, 10, 15, 20, 25, 30		
Nitrogen as ammonium and as nitrate	1 and 4, 4 and 16, 6 and 24		

[a] These nutrient solutions were prepared from Gm. equiv. stock solutions of the following salts, by adding 1.0 ml. of the stock solution for each mg. equiv. desired in the final solution to water, and made up to the desired volume. Calcium nitrate, potassium nitrate, potassium acid phosphate (Sorensen's), magnesium sulfate, ammonium chloride, ferric citrate (0.5%). Where cations were added without nitrate the corresponding chloride was used, since chloride has not been proved essential for plant growth.

Measurements of shoot length, number of initiated and elongated leaves, flowering shoots, and lateral vegetative shoots were made weekly during the growth period. Plants were harvested at a selected stage, i. e., when 50% of the flower buds of the plants at a particular nitrogen level had opened. Fresh weights were taken at harvest on all plants. Representative samples were taken for the various nitrogen determinations and all other plants were dried (air dried for several days, then two hours at 100°) for dry weights. Samples of the nutrient solutions were analyzed after harvesting to determine actual uptake of nitrogen by the plants.

EXPERIMENTAL RESULTS

There was a regular progression of growth increases with increased nitrogen supply even to the maximum used (Fig. 1 and Table II). This would indicate that the plants might properly use nitrate or ammonia in the greater amount, though the dry weights of leaves and shoots (Table III) indicate that at the upper level of nitrogen supply less photosynthetic products are accumulated, rendering nitrogen levels higher than that which was used undesirable.

The regular progression of shoot elongation with time was of the same order of magnitude as the fresh weights of the plants at the time of harvest. In each case the NH_4-NO_3 plants were somewhat larger than those of the corresponding level of NO_3.

The number of leaves produced appeared to be independent of changes in the nitrogen supply, since growth involved production of leaves by apical meristems and the pattern of elongation which followed the leaf initiation was the phas that reflected the nutritional differences. However, branching and flowering (Table IV) were markedly influenced by the nitrogen source. At high nitrogen levels, particularly NH_4-NO_3, the number of branches produced and the number of flowers per branch were greatly increased (50 flowers *vs.* 20–30

Fig. 1.—Plants of *Atropa belladonna* L. grown in nutrient solutions that contained (upper row) 5, 10, 15, 20, 25, 30 mg. equiv./L. of nitrate nitrogen, and (lower row) 5, 20, 30 mg. equiv./L. of ammonia-nitrate nitrogen.

TABLE II.—FRESH WEIGHT OF SEPARATED PARTS OF BELLADONNA PLANTS[a]

Solution Composition, mg. equiv. of N/L.	Leaf	Stem	Root	Flowers	Total Plant
			Nitrate		
5	20.5	33.6	26.8	4.6	85.5
S. D.	5.2	12.8	8.6	3.3	29.7
10	32.1	43.0	27.2	5.9	108.2
S. D.	14.3	19.1	9.0	3.4	39.2
15	39.6	46.1	33.1	6.7	125.5
S. D.	13.1	18.6	2.5	4.4	39.6
20	43.1	46.2	33.5	7.6	130.4
S. D.	10.9	15.7	9.9	5.4	41.4
25	48.0	46.6	33.5	6.6	134.7
S. D.	11.0	18.0	14.1	5.1	46.1
30	51.9	48.0	36.2	5.7	141.8
S. D.	15.0	18.0	18.6	4.2	53.1
			Ammonia-Nitrate		
5	19.0	31.1	30.1	4.9	85.1
S. D.	5.3	12.1	12.0	2.4	30.8
20	51.6	55.8	36.7	9.1	153.2
S. D.	13.8	17.7	10.5	5.2	43.9
30	57.8	61.2	40.1	12.8	171.9
S. D.	10.4	13.6	12.2	5.5	35.8

[a] Weights in Gm. are means of 12 plants.

TABLE III.—DRY WEIGHT OF SEPARATED PARTS OF BELLADONNA PLANTS[a]

Solution Composition, mg. equiv. of N/L.	Leaf	Stem	Root	Flowers	Total Plant
			Nitrate		
5	2.41	5.84	3.10	0.88	13.26
S. D.	0.53	0.50	0.25	0.11	0.45
10	4.20	8.43	3.15	1.30	17.08
S. D.	1.39	1.02	0.68	0.30	1.50
15	6.33	8.43	3.57	1.40	19.73
S. D.	0.52	1.03	0.36	0.24	1.45
20	7.32	9.23	3.92	1.58	22.05
S. D.	0.81	1.01	0.58	0.42	1.09
25	6.02	8.11	3.37	1.32	18.82
S. D.	0.44	2.72	0.63	0.79	4.05
30	6.46	7.96	3.65	1.23	19.30
S. D.	0.96	0.47	0.42	0.22	1.53
			Ammonia-Nitrate		
5	3.08	5.12	3.69	1.00	12.85
S. D.	0.76	0.63	0.39	0.20	1.74
20	7.44	10.46	3.98	1.82	23.70
S. D.	0.49	0.56	0.31	0.38	0.72
30	7.73	11.40	4.27	2.40	25.80
S. D.	0.61	0.36	0.54	0.64	1.34

[a] Weights in Gm. are means of 12 plants.

at lower nitrogen levels), reflecting the essential need for the constituents of protein in the synthesis of new tissues. Regular increase of fresh weight to the highest level of both nitrate and nitrate-ammonia treatments in all plant parts except flowers, further indicates the high requirement of nitrogen for satisfactory plant synthesis. The reduced weight of flowers at high nitrate indicates that high nitrogen favors vegetative over reproductive growth.

Dry weights varied between 15 and 20% of fresh weights (Tables II, III). At low NO_3 and NH_3-NO_3 levels nutritional effects were similar on fresh and dry weight bases. At high NO_3 levels the dry weight of plant produced was substantially less than that comparable with the fresh weight, indicating that high NO_3 tends to produce a succulent plant. To a lesser extent the same is true of the dry weight of NH_3-NO_3 grown plants. Difference in dry weights of roots between treatments were much less; in fact, the total difference between all treatments was slight, indicating that succulence is a condition of the overground parts.

Increases in per cent moisture content were manifested mainly in the leaves, where the moisture content varied between 82 and 87%. Plants having low water content matured earlier than the more succulent plants.

The ratio of leaf/stem (Table V) varied between 0.6 at low nitrate and 1.0 at high nitrate levels. The comparable ratios where NH_3-NO_3 were used were all lower, indicating that nitrate tends to stimulate leaf development while ammonium ion tends to stimulate stem development. The pattern of leaf/root ratios followed the same direction as leaf/stem, but the magnitude was higher. At low nitrogen levels the plant synthesizes root in preference to leaf and stem tissue. The ratio of leaf to the total plant was so uniform as to indicate the belladonna plant is most strikingly responsive to nutritional treatments through its leaves. Where plants were furnished adequate nitrogen, i. e., 10 mg. equiv. or more of either NO_3 or NH_3-NO_3 the following decimal fractional parts constitute the belladonna plant:

	Fresh Weight	Dry Weight
Leaf	0.35	0.38
Stem	0.35	0.40
Flowers	0.05	0.04
Root	0.25	0.18

DISCUSSION

The water culture method lends itself well to growth of the belladonna plant, where all nutritional variables can be controlled and growth can be measured periodically without sacrificing the plants.

These plants grew well in the greenhouse under such conditions to full maturity, within a period of fifty-five days, without aeration of roots, which compares favorably with the growth cycle of field-grown plants. They differed from field-grown plants mainly in that lateral branching occurred to a lesser degree. By contrast, branching discussed in this work involved branches formed at the shoot apex as a result of floral initiation, and elongation of these branches became inflorescences. This type of branching also occurs with field-grown plants.

After a short period of growth, uptake of ions by the plant caused the hydrogen ion concentration to change, for which adjustment to pH 5.8 was made weekly. The drift was invariably toward the alkaline range, due to preferential uptake of NO_3. During periods of rapid growth and salt uptake the solution pH shifted as high as to pH 7.5; but most adjustments involved not more than 1.5 pH units. Occasionally iron citrate was added in quantities greater than 1 ml. per week when incipient chlorosis indicated a need for iron.

Growth data were based on the means of 12 plants per treatment, allowing statistical support of differences where applicable.

TABLE IV.—NUMBER OF APICAL BRANCHES AND FLOWERS OF BELLADONNA PLANTS[a]

Solution Composition, mg. equiv. of N/L.	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Branches	2	2.8	3.9	4.2	3.5	4.4	2	4.7	7.9
Flowers	16.0	22.5	24.0	30.0	25.0	28.0	18.0	35.0	48.5

[a] Numbers are means of 12 plants.

TABLE V.—RATIOS (FRESH WEIGHT) OF PLANT PARTS OF THE BELLADONNA PLANT

Solution Composition, mg. equiv. of N/L.	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf/stem	0.61	0.76	0.86	0.96	1.00	1.01	0.61	0.96	0.97
Leaf/root	0.77	1.21	1.21	1.30	1.47	1.43	0.62	1.43	1.48
Leaf/total plant	0.24	0.29	0.31	0.34	0.36	0.36	0.23	0.33	0.33

Since NO_3 must be reduced to NH_2 and then to amino acids by amination within the plant tissue, it is to be expected that the NH_2 source would shorten the enzymatically controlled synthetic process.

The number of expanded leaves increased only slightly with increased NO_3, indicating that it is the function of the organized meristem to cut off leaf initials at a fairly uniform rate, and that nutritional variables exert a far more pronounced effect on the rate of elongation of the produced initial than on its rate of production. At high levels of NO_3 the normal floral initiation of shoot apex is curtailed and the vegetative condition is enhanced, thereby producing the well-known succulent condition in plants. The production of dry weight with NH_2-NO_3 was significantly greater than that with NO_3 alone, indicating that increased water in tissue did not occur. This would be due to the fact that the tissue does not accumulate NH_2 in great excess over its metabolic needs as it does NO_3.

The ratio of leaf to root and stem to root under low nitrogen supply point to the larger root system developed under these conditions. Ammonia-NO_3 at adequate levels appears to stimulate stem development as indicated by leaf to stem and stem to root ratios.

SUMMARY

1. Belladonna plants were grown from seed to a selected stage of flower maturity for each of a series of nutritional treatments in water culture over a range of NO_3 and NH_2-NO_3 sources with all of the other known essential elements present at a constant adequate level.

2. Twelve plants were grown to a selected stage of flower maturity for each nutritional treatment which furnished sufficient experimental material to replicate satisfactorily all of the chemical tests that were performed.

3. Growth was characterized by the effect of these variables on shoot length, fresh and dry weight of all plant parts, degree of branching, leaf initiation, flower formation, and the ratio of plant parts to each other.

4. By most of the criteria observed, growth of the plant was better with the NH_2-NO_3 source than with NO_3 alone. On the basis of dry weight produced, the most satisfactory growth was obtained from NO_3 at 20 mg. equiv. and from NH_2-NO_3 at 30 mg. equiv.

5. High levels of NO_3 tend to raise the tissue water level, producing a more succulent plant than that furnished NH_2-NO_3. Because of this variation the dry weight is a more reliable criterion for assessing growth changes.

6. The root system was unusually large under low levels of nitrogen.

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Nitrogenous Constituents of *Atropa belladonna* L. Grown on Different Sources of Externally Supplied Nitrogen*

By L. J. SCHERMEISTER†, F. A. CRANE, and R. F. VOIGT

Nitrates, nitrites, ammonia, amides, amino acids, proteins, and alkaloids were determined in plants under all treatments as previously described. At low nitrogen supply, belladonna synthesized alkaloids preferentially over protein. At high levels more protein and soluble nitrogenous compounds accumulated in tissue while the alkaloid level was relatively low. There appeared to be no direct relation between alkaloid and protein. γ-Aminobutyric acid may be a precursor to the belladonna alkaloids based on amounts of each present.

PLANTS grown by methods explained in the previous report (1) were harvested and prepared for the determination of the various nitrogenous constituents mentioned below. Samples were taken that would provide fresh and dry weights of each plant part, total nitrogen, nitrate, ammonia, amides, amino acids both free and combined in protein, and alkaloids of the various parts.

PROCEDURE

Samples for total nitrogen (Kjeldahl) were taken directly from fresh tissue to avoid nitrogen loss on drying. Tissues used for the determination of nitrate, nitrite, ammonia, and amides were frozen at −20° from the fresh plant at the time of harvest. Samples used for amino acid determination were placed in sufficient 95% ethyl alcohol that with the water normally present in the tissue resulted in a 75% alcohol concentration. If more alcohol was needed to cover tissue, 75% alcohol was added. The remaining tissue was dried for alkaloid determinations.

Ammonia, amide, nitrite, and nitrate fractions were determined by the method of Varner, Bulen, Vanecko, and Burrell (2) which separates these fractions by a combination of Dowex resins and selective pH ranges. This releases the fractions individually so that they can be distilled and titrated in a semimicroKjeldahl apparatus.

Free amino acids and those combined in protein (after acid hydrolysis) were determined by paper chromatography made quantitative by Thompson and Steward (3). Washed and buffered (pH 7.0) sheets of Whatman No. 1 filter papers were used, making reproducibility such that multiple determinations were never more than 5% from each other.

Alkaloids were determined by the method of Brown, Kirch, and Webster (4) with the following variations: (*a*) a 3-Gm. rather than a 10-Gm. sample was employed; (*b*) the material was extracted with 3 ml. of stronger ammonia test solution U. S. P. and 6 ml. of ether, U. S. P., instead of 10 ml. and 20 ml., respectively; (*c*) the total extractive was employed in the chromatographic separation of the alkaloid, instead of a 10-ml. aliquot; (*d*) the total alkaloids were titrated electrometrically to the end point of pH 6.5 instead of using the methyl red indicator.

Total nitrogen in each tissue was determined by the Kjeldahl method described in the U. S. P. XIV (5), including the following modifications: (*a*) A 1-Gm. sample of fresh tissue containing portions from exactly the same areas (apical, median, and basal) of the respective plant parts at the same stage of development were placed in the sulfuric acid digesting mixture and quantitatively transferred into semimicroKjeldahl digestion flasks by rinsing with distilled water. (*b*) The rinse water was evaporated to approximately 5 ml. over a Bunsen burner. After the digestion flask had cooled to room temperature, 0.5 ml. of 30% hydrogen peroxide was added. The sample was digested until the solution acquired a clear bluish green color. (*c*) To absorb heat produced upon addition of sodium hydroxide, the digestion flask was immersed in a cold water bath. (*d*) Fifteen milliliters of 10 *N* sodium hydroxide containing two drops of methyl red indicator was cautiously added, while a few bubbles of steam were coming over from the generator, insuring immediate mixing of the sulfuric acid and the sodium hydroxide. When all of the sodium hydroxide had been added, distillation with steam was begun at once. (*e*) Ten drops of the indicator used by Ma and Zuazaga (6) and 25 ml. of 2% boric acid were placed in the receiving flask.

The ammonia collected in the receiving flask was titrated according to the official U. S. P. method and total nitrogen calculated.

RESULTS

Nitrogenous Constituents

Total Nitrogen.—The belladonna plant follows the commonly reported pattern of absorbing nitrogen (Tables I and II) from a source far in excess of its growth requirements. The greatest absorption was in tissues furnished nitrate (23 mg. nitrogen per Gm. of fresh tissue), but since the NH_2-NO_3 plants grew more vigorously, the amount of nitrogen per total plant exceeded that of the plants furnished

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TABLE I.—TOTAL NITROGEN OF BELLADONNA PLANTS (KJELDAHL)[a]

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	3.98	4.15	4.40	5.63	6.60	5.50	3.16	4.67	5.77
Stem	1.37	2.10	2.14	2.58	3.14	3.02	0.92	3.17	3.56
Root	1.72	2.43	2.48	2.75	3.10	3.32	1.57	2.66	2.73
Flowers	5.40	6.75	7.06	7.26	8.00	10.10	5.34	7.05	6.77

[a] Figures are mg. total nitrogen/Gm. of tissue, fresh weight.

TABLE II.—ABSOLUTE AMOUNTS OF TOTAL NITROGEN[a]

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	50	20	30
Leaf	81.0	133.2	174.2	243.0	316.8	285.4	58.9	241.0	335.7
Stem	46.0	90.3	98.7	119.2	146.3	144.9	28.6	176.9	217.8
Root	46.1	66.1	82.1	92.2	103.8	120.0	47.3	99.1	109.5
Flower	24.8	39.8	47.3	55.1	52.8	57.6	26.2	64.6	87.1
Total plant	197.9	329.4	402.3	509.5	619.7	607.9	161.0	581.6	750.1

[a] Figures are mg. total nitrogen per plant part.

TABLE III.—NITRATE, NITRITE, AMMONIA, AND AMIDE NITROGEN OF BELLADONNA PLANTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

Soln. Compn., mg. Equiv. Nitrogen/L.		Nitrate						Ammonia-Nitrate		
		5	10	15	20	25	30	5	20	30
Nitrate determined in tissue	Leaf	0.031	0.030	0.042	0.071	0.195	0.274	0.031	0.050	0.064
	Stem	0.030	0.050	0.166	0.280	0.257	0.267	0.032	0.121	0.055
	Root	0.030	0.045	0.050	0.077	0.152	0.162	0.032	0.048	0.062
Nitrite determined in tissue	Leaf	...	...	...	...	...	...	...	...	...
	Stem	...	...	...	...	...	...	...	...	0.03
	Root	...	...	...	...	...	...	...	...	0.03
Ammonia determined in tissue	Leaf	...	...	...	0.015	0.016	0.018	...	0.013	0.018
	Stem	...	0.063	0.073	0.124	0.182	0.184	...	0.184	0.218
	Root	...	...	...	0.020	0.025	0.030	...	0.023	0.025
Amide determined in tissue	Leaf	0.034	0.059	0.061	0.070	0.071	0.064	0.041	0.070	0.080
	Stem	0.034	0.122	0.176	0.284	0.315	0.410	0.046	0.311	0.382
	Root	0.036	0.026	0.039	0.050	0.062	0.090	0.029	0.028	0.032

[a] Nitrogen, mg./Gm. fresh weight.

nitrate alone. Flower tissue contained the highest concentration of total nitrogen on a "per Gm. of tissue" basis but the amount of tissue was small.

Nitrate and Ammonia-Nitrate.—These were absorbed from solutions by the plants at all levels (Table III). At the lower levels of nitrate and at all levels of NH_4-NO_3 the order of absorption indicates its direct use without accumulation in the tissue. At higher NO_3 levels there was clear evidence of accumulation in the tissue far beyond the ability of the plant to metabolize it.

Nitrite.—Although nitrites in relatively small concentrations are considered toxic and do not generally accumulate in plants, detectable amounts (0.03 mg./Gm.) did occur in stem and root tissues of belladonna at 30 mg./equiv. of NH_4-NO_3. This can be interpreted as an inability of the plant to convert readily all the available nitrate into ammonia via nitrite because of the concentration of ammonia already present in these tissues.

Ammonia Nitrogen.—Detected chiefly in stems at the higher levels of nitrate and especially NH_4-NO_3 supply, this indicates that ammonium is commonly utilized in tissue so rapidly that it is detected only in the area of translocation.

Amides.—Like ammonia, these are metabolized quickly in leaf and root and are present in an appreciable quantity in stem. The presence of the major amides was detected along with the amino acids, chromatographically.

Amino Acids and Amides.—Those present in the alcohol-soluble fraction (free amino acids) (Tables IV, V, and VI) include cysteic acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, the leucines, and γ-aminobutyric acid. Associated with increasing nitrate supply are greatly increased amounts of aspartic acid, glutamic acid, glutamine, and proline. Plants grown at the higher NH_4-NO_3 levels contained 50% more soluble nitrogen in the leaf (Table IV) than the corresponding NO_3 examples, the difference being made up of larger quantities of glutamic acid, glutamine, proline, and the presence of significant quantities of asparagine that were not present in the nitrate grown plants.

It is noteworthy that glutamine as a storage form of nitrogen increased up to 25 mg. equivalents of NO_3. Above this level a greater proportion of the nitrogen was converted into protein.

Asparagine, which accumulated at the upper levels of ammonium was not present at any level of nitrate supply. This bears out the idea of Steward and Preston (7) that glutamine is intimately associated with nitrogen synthesis and asparagine is associated with protein hydrolysis in the tissue.

It is surprising that a closed ring heterocyclic

TABLE IV.—COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solutions, mg. Equiv./Liter								
	NO_3						NO_3-NH_4		
Amino Acids	5	10	15	20	25	30	5	20	30
Cysteic from cysteine	...	10.0	20.0	25.0	30.0	30.0	...	30.0	30.0
Aspartic	60.0	40.0	230.0	90.0	265.0	260.0	25.0	245.0	190.0
Glutamic	13.6	12.4	58.5	85.0	85.6	52.0	40.3	100.0	99.1
Serine	3.4	...	41.3	30.0	34.1	21.9	61.2	58.4	26.0
Glycine	14.4	23.6	40.7	24.6	31.9	46.5	13.0	62.9	61.2
Asparagine	...	...	...	...	...	...	...	130.0	150.0
Threonine	...	43.0	25.3	14.0	30.6	34.8	33.1	51.8	23.7
Alanine	47.5	70.7	55.2	76.3	113.3	53.4	70.0	175.2	142.6
Glutamine	34.0	37.5	284.6	375.0	472.5	270.0	110.0	1,375.0	655.0
Proline	333.0	531.6	600.0	644.0	1,010.0	789.0	133.0	1,147.0	1,554.0
Valine	14.7	18.9	27.9	17.5	20.3	11.4	23.3	35.5	23.1
Leucines	27.6	31.4	27.7	20.3	21.1	21.6	47.4	45.3	30.9
γ-Aminobutyric	72.2	104.5	161.5	150.0	167.3	90.1	150.8	305.0	200.0
Total, mcg./Gm.	620.4	950.0	1,572.0	1,551.0	2,281.0	1,680.0	708.0	3,760.0	3,182.0

[a] Amino acid, mcg./Gm. fresh weight.

TABLE V.—COMPOSITION OF THE ALCOHOL-SOLUBLE NITROGEN FRACTIONS OF BELLADONNA STEM AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solution, mg. Equiv./Liter								
	NO_3						NO_3-NH_4		
Amino Acids	5	10	15	20	25	30	5	20	30
Cysteic from cysteine	...	...	25.0	40.0	35.0	35.0	...	34.0	37.0
Aspartic	90.0	92.0	130.0	200.0	190.0	210.0	100.0	160.0	290.0
Glutamic	28.4	31.2	34.2	66.0	53.1	51.2	12.0	53.9	36.4
Serine	14.2	28.0	22.9	35.7	26.0	38.2	10.1	33.9	19.3
Glycine	29.3	53.6	39.9	41.2	37.8	66.3	28.3	77.2	88.2
Asparagine	112.0	382.0	682.0	814.0	786.0	838.0	...	505.0	850.0
Threonine	9.2	46.8	28.8	58.3	45.5	44.5	19.8	64.1	60.5
Alanine	22.5	35.6	17.4	35.9	36.8	24.5	15.6	25.3	25.3
Glutamine	245.0	2,141.0	3,606.0	3,080.0	3,187.0	3,025.0	240.7	5,300.0	8,905.0
Proline	692.0	1,055.0	757.0	940.0	1,082.0	1,212.0	717.0	890.0	1,200.0
Valine	29.7	32.2	23.4	19.1	30.0	33.5	50.5	38.5	40.9
Leucines	64.3	131.7	47.2	45.3	46.7	70.4	83.9	84.5	87.1
Phenylalanine	65.0	177.0	71.8	63.5	77.5	100.7	58.5	63.6	65.6
Tyrosine	45.4	91.5	15.1	25.5	33.3	29.9	48.1	31.1	31.3
β-Alanine	...	...	22.2	34.1	30.0	20.0	...	15.8	17.0
γ-Aminobutyric	55.3	58.4	42.5	59.7	64.0	71.4	54.3	82.8	59.7
Total, mcg./Gm.	1,501.0	4,356.0	5,467.0	5,556.0	5,760.0	5,870.0	1,438.0	7,459.0	11,813.0

[a] Amino acid, mcg./Gm. fresh weight.

TABLE VI.—COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA ROOT AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solutions, mg. Equiv./Liter								
	NO_3						NO_3-NH_4		
Amino Acids	5	10	15	20	25	30	5	20	30
Aspartic	52.3	51.8	80.4	70.6	170.6	286.3	33.3	243.0	230.0
Glutamic	3.0	28.7	28.2	83.6	133.6	77.3	2.2	140.0	222.0
Serine	...	4.4	9.4	20.9	16.2	24.3	...	27.5	28.3
Glycine	6.0	16.7	7.9	24.2	29.7	37.1	...	58.0	112.9
Asparagine	...	...	...	...	...	...	...	84.0	535.0
Threonine	...	5.2	10.5	12.8	15.2	33.1	...	29.5	24.5
Alanine	3.4	13.3	10.6	33.1	36.1	83.0	4.6	56.9	50.2
Glutamine	41.6	43.6	30.0	45.2	102.2	131.0	54.0	2,676.0	5,002.0
Proline	...	...	...	...	172.3	230.9	...	172.0	390.0
Valine	4.6	14.7	7.4	7.1	22.1	14.6	2.9	16.0	16.9
Leucines	...	13.0	10.3	7.7	13.1	10.1	6.5	18.6	23.1
Phenylalanine	...	15.5	...	8.3	9.2	8.8	8.9	27.7	...
γ-Aminobutyric	21.0	189.0	21.2	120.4	56.8	183.4	16.2	222.0	163.0
Total mcg./Gm.	131.9	396.4	215.9	433.9	777.1	1,119.9	128.6	3,771.0	6,798.0

[a] Amino acid, mcg./Gm. fresh weight.

amino acid such as proline should have constituted over 40% of the soluble nitrogen at all levels.

The total soluble nitrogen produced was greater at each level of ammonia-nitrate than with nitrate-nitrogen alone. The major portion of the increase was due to the amides, asparagine, and glutamine at the higher nitrogen levels. The appearance of asparagine at the higher levels of ammonia-nitrate was accounted for by a reduction in the amount of aspartic acid in this series when compared with that supplied with nitrate. The combination of glutamic acid and glutamine under ammonia nutrition was more than three times that under the comparable nitrate nutrition, whereas the combination of aspartic acid and asparagine was roughly the same under both treatments.

The total amount of solable nitrogen present in the stem (Table V) was approximately three times that found in the leaf. Aspartic and glutamic acids increased in the same proportions throughout the treatments, i. e., the aspartic acid was about three times the glutamic acid in most cases. Their amides also increased throughout the treatments but by contrast, glutamine was about three times the asparagine in all cases.

Phenylalanine, tyrosine, β-alanine were not detected in leaf tissue but were present in quantitative amounts in the stem.

The total amount of soluble nitrogen present in the roots (Table VI) that had been furnished nitrate was only half that present in the leaf tissue, but roots furnished the high level of NH_3-NO_3 contained more than twice the soluble nitrogen of their leaves. This large amount of soluble nitrogen was predominantly asparagine and glutamine, indicating that the ammonia source stimulated the accumulation of amide in root tissue. γ-Aminobutyric acid is a major fraction in root tissue, particularly at the high levels of both NO_3 and NH_3-NO_3. It was observed that an abundance of γ-aminobutyric acid seemed to be associated with the presence of large amounts of alkaloid in the root.

Alcohol-Insoluble Nitrogen (Protein).—Although the nutrient supply had a pronounced effect on the metabolism of all plant parts, relatively slight changes in the amino acid composition of their protein was observed. (Compare Tables IV and VII, V and VIII, VI and IX.) All of the amino acid's present in the soluble fraction except γ-aminobutyric acid occurred much more abundantly in the protein than in the free state. The ratio of aspartic acid to glutamic acid in the leaf protein varied from 2:1 to 3:1 with the greater ratio being associated with the higher nitrogen levels. Serine, glycine, threonine, alanine, valine, phenylalanine, and tyrosine remained substantially constant throughout all of the treatments. All levels of ammonia-nitrate produced more protein than the corresponding nitrate treatment. Lysine and arginine commonly found in the protein fraction of plants were not detected in either the alcohol-soluble or insoluble fractions of the belladonna plant.

The composition of the stem protein (Table VIII) was similar to that of the leaf protein, but amounted to only one-tenth that of the leaf except at lowest NO_3 levels. The ratio of aspartic acid to glutamic acid in the NO_3 series was 2:1; in the NH_3-NO_3 it was 1:1, indicating that NH_3 tends to stimulate the production of glutamic acid over aspartic acid.

The protein synthesized in the root (Table IX) contained the compounds previously mentioned in leaf and stem and contained, in addition, cysteic acid in all treatments. It is surprising that this is the only sulfur-containing amino acid found in the protein fraction. The amount of protein synthesized in roots was about one-forth that present in leaf and slightly more than twice that in stem. The compounds most noticeable in the root protein were the leucines, tyrosine, proline, and hydroxyproline. Of these, hydroxyproline is present in greater quantity than in any other plant part.

TABLE VII.—COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solution, mg. Equiv./Liter								
	NO_3						NO_3-NH_4		
Amino Acids	5	10	15	20	25	30	5	20	30
Cysteic from cysteine	...	...	...	...	...	...	...	...	...
Aspartic	2,140	3,000	5,652	2,601	5,690	6,584	5,242	3,536	3,905
Glutamic	970	1,000	2,314	1,910	1,993	1,960	2,065	1,852	2,009
Serine	185	800	1,291	296	998	834	974	1,310	1,988
Glycine	430	700	1,045	679	882	919	550	596	1,865
Asparagine	...	...	...	...	...	...	...	...	...
Threonine	910	1,100	1,394	1,240	1,159	1,100	1,099	1,352	1,606
Alanine	648	1,050	1,842	2,125	2,170	1,439	1,077	2,909	2,046
Glutamine	...	...	...	...	...	...	...	...	...
Proline	730	1,200	2,883	1,425	3,000	3,952	1,319	4,660	4,470
Valine	1,175	1,425	1,833	1,668	1,880	2,100	1,391	2,490	2,625
Leucines	2,310	2,700	3,766	3,070	4,067	4,645	3,312	6,577	5,551
Phenylalanine	1,010	1,220	1,428	1,363	1,388	1,440	...	1,979	2,139
Tyrosine	747	570	553	503	631	744	690	930	695
β-Alanine	...	...	...	...	...	...	...	...	...
γ-Aminobutyric	...	...	...	...	...	...	...	...	...
OH Proline	...	...	266	175	247	184	276	552	423
Total mcg./Gm.	11,355	14,765	24,267	17,055	24,105	25,900	17,995	28,743	29,322

[a] Amino acid, mcg./Gm. fresh weight.

TABLE VIII.—COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* STEM AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solution, mg. Equiv./Liter								
	NO_3						NO_3-NH_3		
Amino Acids	5	10	15	20	25	30	5	20	30
Cysteic from cysteine	...	...	...	...	...	...	...	...	...
Aspartic	450	476	508	656	417	531	525	593	519
Glutamic	168	257	296	390	244	245	240	462	429
Serine	...	112	103	125	58	103	76	132	131
Glycine	59	104	129	145	93	149	66	129	120
Asparagine	...	...	...	...	...	...	...	...	...
Threonine	110	100	142	187	77	124	47	53	50
Alanine	135	176	191	221	187	145	161	158	183
Glutamine	...	...	...	...	...	...	...	...	...
Proline	25	303	249	541	294	37	20	325	625
Valine	247	240	229	282	237	238	185	250	245
Leucines	285	398	225	512	431	413	380	431	425
Phenylalanine	150	175	188	250	193	112	165	181	263
Tyrosine	...	...	78	120	119	95	82	97	104
β-Alanine	...	...	...	...	...	...	...	...	...
γ-Aminobutyric	...	...	...	...	...	...	...	...	...
OH Proline	...	140	139	171	75	75	...	165	153
Total mcg./Gm.	1,630	2,481	2,478	3,600	2,425	2,266	1,947	2,976	3,247

[a] Amino acid, mcg./Gm. fresh weight.

TABLE IX.—COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* ROOT AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS[a]

	Nitrogen in Nutrient Solution, mg. Equiv./Liter								
	NO_3						NO_3-NH_3		
Amino Acids	5	10	15	20	25	30	5	20	30
Cysteic from cysteine	220	270	300	260	262	260	220	240	250
Aspartic	594	682	1,456	543	560	403	449	608	722
Glutamic	226	232	650	672	622	662	273	253	453
Serine	106	123	594	336	269	248	130	350	341
Glycine	177	255	360	299	269	208	163	575	351
Asparagine	...	...	...	...	...	...	...	...	...
Threonine	220	204	636	368	345	338	332	525	360
Alanine	270	255	385	391	356	351	291	490	361
Glutamine	...	...	...	...	...	...	...	...	...
Proline	428	272	1,540	634	1,189	1,182	695	1,291	2,070
Valine	321	528	666	585	529	522	528	717	667
Leucines	490	773	1,143	1,026	899	880	843	1,322	1,176
Phenylalanine	285	403	428	381	426	429	317	469	426
Tyrosine	130	310	316	286	242	278	225	300	317
β-Alanine	...	...	...	...	...	...	...	...	...
γ-Aminobutyric	...	...	...	...	...	...	...	...	...
OH Proline	376	570	459	550	522	663	429	653	645
Total mcg./Gm.	3,843	4,877	9,943	6,331	6,490	6,404	4,895	7,793	8,139

[a] Amino acid, mcg./Gm. fresh weight.

Alkaloids.—Regardless of the amount of nitrate furnished, the amount of alkaloid found in the leaf was approximately 1.5 mg./Gm. dry weight (Tables X and XI). It indicates that inorganic nitrogen supplied in minimal amounts or more has little effect on the per cent of alkaloid in the leaf. There was a regular progression of absolute amounts of alkaloid in the leaf tissue with increasing nitrate concentration. Since the rate of alkaloid synthesis was fairly constant, this progression is taken to be largely an indication of the rate of tissue production.

The relative amount of alkaloid contained in stem tissue was roughly three-fourths that present in the leaf. The notable exception was 30 mg. equiv. where alkaloid content was as high as leaf alkaloid. The same relationship applied to alkaloid synthesis under ammonia nutrition.

The notable feature of alkaloid synthesis was its high relative concentration in root tissue. This was unusually striking at 5 mg. equiv. of nitrate where 3.0 mg. of alkaloid were present per Gm. of dry tissue. This indicates that nitrogen was used in alkaloid synthesis in preference to that available for protein synthesis and growth. The absolute amounts of alkaloid present in root closely approximated that of leaf and stem combined. This was obviously due to the larger amounts of dry weight in leaf and stem.

Alkaloid synthesis under NH_3 nutrition exceeded that of plants furnished NO_3 at the higher nitrogen levels of both relative and absolute bases. These facts indicate that alkaloid synthesis has taken place in the root and the product was then translocated to overground plant parts. It is important to note that where the larger amounts of alkaloid were present in root, greater amounts of γ-aminobutyric

TABLE X.—TOTAL ALKALOIDS OF BELLADONNA/GM. OF DRY TISSUE[a]

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	1.61	1.73	1.45	1.58	1.67	1.66	1.86	1.53	1.57
Stem	1.06	0.91	1.10	1.05	1.14	1.48	1.34	1.16	1.23
Root	3.04	1.76	2.52	2.90	3.09	2.50	2.86	4.02	3.90
Flowers	1.16	1.26	1.50	1.60	1.64	1.97	1.30	1.46	1.68
Total plant, av. mg./Gm.	1.72	1.41	1.64	1.78	1.88	1.90	1.84	2.04	2.09

[a] Figures are mg. of total alkaloids.

TABLE XI.—ABSOLUTE AMOUNTS OF TOTAL ALKALOIDS[a]

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	5.54	7.27	9.18	11.57	10.05	10.72	5.65	11.38	12.14
Stem	6.19	7.67	9.27	9.69	9.25	11.78	6.86	12.13	14.02
Root	9.42	5.54	9.00	11.36	10.41	9.13	10.55	16.00	16.65
Flowers	1.02	1.64	2.10	2.53	2.16	2.42	1.30	2.66	4.03
Total plant	22.80	24.08	29.55	35.15	31.87	34.05	24.36	42.17	46.84

[a] Figures are mg. of total alkaloids per plant part.

TABLE XII.—TOTAL NITROGEN IN THE NITROGENOUS FRACTIONS OF THE LEAF, STEM, AND ROOT OF *Atropa belladonna* L.[a]

Solution Compn., mg. Equiv. N/L.	NO_3						NH_3-NO_3		
	5	10	15	20	25	30	5	20	30
Nitrate (nitrogen)	2.50	4.77	11.06	19.40	26.29	32.95	2.52	11.15	9.61
Nitrite (nitrogen)	...	...	...	...	...	...	...	...	4.69
Ammonia (nitrogen)	...	2.71	3.36	7.07	10.23	11.41	...	11.84	15.39
Amide (nitrogen)	1.74	23.89	41.25	40.32	42.67	41.93	2.61	100.85	175.29
Soluble amino acid (nitrogen)	11.05	36.99	52.22	54.82	64.79	65.91	10.23	130.60	208.32
Protein (nitrogen)	62.96	11.85	216.99	177.61	235.25	269.24	86.61	277.35	355.20
Alkaloid (nitrogen)	0.87	0.99	1.30	1.56	1.40	1.51	1.11	1.90	2.04
Total mg. of N fractions	77.38	157.31	284.93	260.46	337.96	381.02	100.67	432.84	595.25
Total N by Kjeldahl	173.00	279.00	373.70	451.70	528.00	547.00	161.00	515.00	665.00
Total nitrogen furnished per plant	192.00	385.00	578.50	770.00	962.00	1,130.00	192.00	770.00	1,130.00
Total nitrogen remaining in solution after growth period	0.00	0.00	150.00	250.51	312.50	525.00	0.00	180.00	400.00

[a] Numbers are total mg. of nitrogen as the fraction listed per plant, mean of 12 plants.

acid and smaller amounts of proline were present in the soluble nitrogen fractions than that found in the other plant parts.

Interrelation of the Nitrogen Fractions.—The nitrogenous compounds that tend to increase with increasing nitrate source are nitrate, ammonia, and free amino acids (Table XII). Those that tend to decrease are amides, protein, and alkaloids. With NH_3-NO_3 supplied plants, nitrate decreased but there was a marked increase of free amino acids. The percentage of protein and greatly increased percentage of free amino acids indicates that at a high ammonia supply the amino acids were not rapidly combined into protein and therefore accumulated as free compounds.

DISCUSSION

At the close of the growth experiments Kjeldahl determinations were made on the nutrient solutions to determine the amount of nitrogen present (Table XII). At the lower levels all of the nitrogen had been taken up by the plant. From 15 mg. equiv. of nitrogen, progressively larger amounts were still present and available. The summation of the various fractions did not equal the total nitrogen determined by the Kjeldahl method because the fractions determined were not exhaustive and, to an extent, overlapped. Because of the small amount of tissue available the nitrogenous fractions of flowers were not determined.

Ammonia taken in by the roots was metabolized directly but at the higher levels was also accumulated. This was almost entirely in the stem. The only places where NO_2 was detected in tissue was in the root, where small amounts were present, and in the stems where larger amounts were observed. These were produced in plants furnished the highest NH_3-NO_3 level. The larger amount of NH_3 already present in tissue probably hindered the conversion of NO_2 to NH_3, causing it to accumulate under these conditions.

The major amino acids occurring in most plant parts included aspartic acid and its amide, asparagine, glutamic acid and its amide, glutamine, and γ-aminobutyric acid, which is thought to be formed by decarboxylation from glutamic acid. Variation among these compounds accounted for the majority of the changes in the soluble nitrogen fraction.

In leaf and root all were present except asparagine on nitrate nutrition, whereas in stem, asparagine was present at all levels of both sources of nitrogen. Levitt (8) has indicated that while glutamine is generally associated with processes involving synthesis, asparagine appears to be related to hydrolysis of protein and storage of soluble nitrogen. Asparagine, which was present on NH_3-NO_3 nutrition, has been described as a method of rendering high tissue concentration of NH_3 innocuous.

Proline was present generally in large amounts in leaf and stem tissue under all nitrogen levels, and accumulated in roots where the alkaloid concentration declined. It appears reasonable that proline could be linked with an oxidized dicarboxylic acid fraction to produce the tropane nucleus. The fact that free proline was present in roots only at the high nitrogen concentration and where alkaloid synthesis was low, or curtailed, would indicate that the proline was used in synthesis at lower nitrogen concentrations, and some unexplained cause was responsible for the blocking of this synthesis at higher nitrogen levels with a consequent accumulation of proline. Hydroxyproline was present in the root protein in quantity where proline concentration increased. The reactions by which proline could be used in the synthesis of hyoscyamine might also be the same for the conversion of hydroxyproline into scopolamine, which is also present. From the data it would appear that the γ-aminobutyric acid might serve as one possible precursor of the alkaloid since its concentration decreased as alkaloid increased, and vice versa. The function of γ-aminobutyric acid is probably a raw material for carboxylation and oxidation reactions in the production of a tropane nucleus. James (9) indicated that the γ type amino nitrogen compound is effective in alkaloid synthesis.

The composition of the plant proteins was much less subject to variation with nutritional treatment than any of the nitrogenous fractions. Since protein is a product of metabolic processes rather than an intermediate, it is to be expected that its degree of stability would be much greater. The largest fraction of leaf and stem protein was aspartic acid, which reached a ratio of 3:1 over glutamic acid at high nitrogen levels, indicating that the belladonna plant prefers the four carbon dicarboxylic acids in its metabolism in preference to the five carbon acids stemming from glutamic acid. At all levels of NH_3 the plant produced more protein than comparable plants furnished nitrate. The advantage gained by furnishing the plant a reduced form of nitrogen appears to have its effect up to and including the synthesis of protein. The small amount of stem protein (one-tenth that of leaf) is explained in the fact that the majority of stem is composed of vascular and structural elements. It is reasonable that the high per cent of soluble nitrogen found in stem was present in these conducting elements—products of leaf and root synthesis in the process of translocation. The protein of the root was similar in composition to that of the leaf but in smaller amount.

The synthesis of alkaloid does not appear directly related to the concentration of simple nitrogenous sources. The assumption that alkaloid is synthesized in root was verified by its high concentration in root at all nitrogen levels. It is surprising that in the translocation to overground parts, the relative amount present in leaf is greater than that in stem. This may be partially explained by the fact that meristematic tissue tends to hold a high concentration of alkaloid. The alkaloid concentration in the flower buds was relatively high, further indicating absorption at points of high metabolic activity. The low concentration in stem is a result of fully differentiated conducting tissue and the small amount of matured storage tissue composing it.

Several workers, James (9), Cromwell (10), and Leete, Marion, and Spencer (11), have hypothesized the use of arginine and ornithine in the synthesis of belladonna alkaloids. The fact that neither of these compounds was detected chromatographically indicates that their importance in direct synthesis must be a minor one. The introduction of exogenous ornithine in excised leaf or root tissue with a consequent occurrence in alkaloid did not demonstrate the path of synthesis employed in the intact plant. Elucidation of the actual pathway of synthesis must be demonstrated with a complete system that is not subject to drastic external constraints on normal equilibria.

Certainly demonstration of the roles that proline, hydroxyproline, and γ-aminobutyric acid play in the synthesis of the belladonna alkaloids is well worth further study along this line.

SUMMARY

Belladonna plants grown on six levels of nitrate and three levels of NH_3-NO_3 were harvested and the following determinations were made on each plant part: nitrate, nitrite, ammonia, amides, free amino acids, protein (following hydrolysis), alkaloids, and total nitrogen.

1. There was a regular progression of NO_3 concentration in the tissue with increasing NO_3 supplied, extending to the point of accumulation far beyond the tissue requirements. The same thing applied to ammonia in a lesser degree only at high levels of NH_3-NO_3 supply.

2. Nitrite was detected in tissue only at highest levels of NH_3-NO_3, indicating its transient condition in nitrogen synthesis.

3. The concentration of NH_3 in tissue was much less than that of NO_3 and was confined in quantity to the stem.

4. The amides were present at high levels of NO_3 and NH_3-NO_3 and amounted in the stem to four times the quantity in leaf and/or root.

5. The free amino acids amounted to one-tenth or more of the total nitrogen present. The following compounds were shown to be present at least in one plant part: cysteic acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline valine, leucine, phenylalanine, tyrosine, β-alanine, γ-aminobutyric acid, and hydroxyproline.

The largest proportion of these compounds is composed of aspartic acid and its amide asparagine, glutamic acid and its amide glutamine, γ-aminobutyric acid, which is assumed to be made from glutamic acid by decarboxylation, and finally proline.

6. The NH_3-NO_3 source stimulated greater formation of the 5-carbon dicarboxylic acids (glutamic and its relatives), while the NO_3 source stimulated greater production of the 4-carbon acids (aspartic and its relatives).

7. The protein fraction was by far the largest, amounting to one-half or more of the total nitrogen. Its composition was fairly constant, being the result of metabolism rather than active metabolites. The protein of leaf and root was similar in composition and markedly different from stem, which served mainly as a storehouse of soluble nitrogen.

8. The alkaloid concentration was greatest in root on the NH_3-NO_3 source, and coincided with small amounts of both free and combined proline. A possible mechanism whereby proline might be used in alkaloid synthesis is suggested. The relative concentration of alkaloid in leaf and flowers was substantially higher than that of stem, suggesting a number of problems involving its translocation.

9. Associated with the large amounts of alkaloid in roots are extremely large amounts of γ-aminobutyric acid, which could well fill the role of a γ semialdehyde as postulated by Vogel and Bonner (12) to be a precursor of alkaloid.

10. The rate of synthesis of nitrogenous fractions indicates protein and alkaloid are both products of a soluble nitrogen pool and are not directly interrelated in their synthesis or hydrolysis. The specific compounds mentioned; proline, hydroxyproline, and γ-aminobutyric acid, appear to be involved in the synthesis of alkaloid more than the majority of the free amino acids which are largely involved in protein synthesis.

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Preparation of 1-Alkyl-2,5-piperazinediones*

By WILLIAM O. FOYE and DOUGLAS H. KAY†

General methods for the preparation of 1-alkyl-2,5-piperazinediones were found in the cyclization of N-alkylglycylglycines and the alkylation of the sodium salt of 1-acetyl-2,5-piperazinedione. 1-Methyl-2,5-piperazinedione showed significant antispasmodic activity.

LITTLE INFORMATION has been reported on the biological activity of 2,5-piperazinediones. Martinez (1) has reported that prior injection of 2,5-piperazinedione protected rats from diabetes caused by alloxan, and the growth of the embryo chick heart was reported to be favored by 2,5-piperazinedione (2). Fischer (3) has reported that 2,5-piperazinedione showed no antibacterial activity against *Staphylococcus aureus*, but 3-methylene-2,5-piperazinedione showed good activity *vs.* this organism. Miura (4) found only a slight bacteriostatic effect against *Trichophyton gypseum* by 2,5-piperazinedione, but the 3,6-dimethyl derivative was synergistic with sulfanilamide against *Escherichia coli*. More recently, de Jongh (5) investigated a series of N,N′-substituted 2,5- and 2,6-piperazinediones and found marked central nervous system depressant action as well as some tranquilizing and antispasmodic activity in some of the compounds.

Since a number of oxygenated heterocycles have shown antispasmodic effects, it was con-

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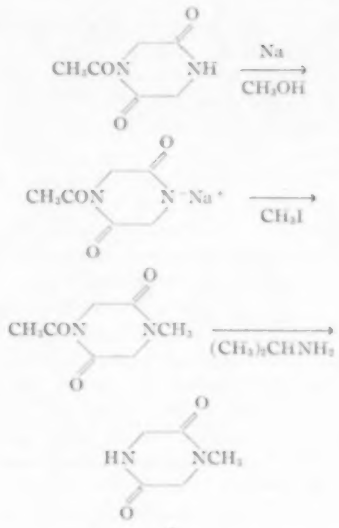
sidered that 2,5-piperazinedione derivatives might be worth while to examine for antispasmodic as well as other pharmacological effects. It was decided to prepare 1-alkyl-2,5-piperazinediones, since derivatives of this type in the piperazine series have shown a variety of pharmacological activities. With the exception of 1-methyl-2,5-piperazinedione, prepared by Chase and Downes (6) by cyclization of sarcosylglycine, no compounds of this type were found in the literature. A general method of preparation for 1-alkyl-2,5-piperazinediones was therefore investigated.

METHOD

The use of several common acylating agents as possible blocking groups for one of the piperazinedione nitrogens was found unsuccessful in providing a reasonably stable monoacyl derivative of 2,5-piperazinedione. Treatment of 2,5-piperazinedione with ethyl chlorocarbonate, for instance, resulted in ring cleavage, and glycylglycine hydrochloride was obtained. Reactions with benzenesulfonyl chloride and ethanesulfonyl chloride also resulted in ring cleavage. Reaction with myristoyl chloride, however, gave both 1-myristoyl- and 1,4-dimyristoyl-2,5-piperazinedione, which were easily separated. Treatment of 1-myristoyl-2,5-piperazinedione with methyl iodide did not give the desired 4-methyl derivative, but instead, 1,4-dimyristoyl-2,5-piperazinedione was produced. Reaction of the 1-myristoyl derivative with diethyl sulfate, however, gave 1,4-diethyl-2,5-piperazinedione, and reaction with benzenesulfonyl chloride likewise gave the 1,4-dibenzenesulfonyl derivative.

1-Acetyl-2,5-piperazinedione was obtained by a modification of Petrova's procedure (7) using an exchange reaction between the 1,4-diacetyl derivative and isopropylamine. The monoacetyl derivative was found to melt at 186–188°, but was shown by mixed melting point to differ from acetylglycylglycine, reported by Fischer (8) to melt at 187–189°. Furthermore, preparation of the acetyldipeptide by Fischer's procedure (8) gave a product melting at 191–192°. Reaction of 1-acetyl-2,5-piperazinedione with acylating and alkylating agents generally resulted in ring opening, however. By first forming the sodium salt of 1-acetyl-2,5-piperazinedione in absolute methanol, alkylation was successful, and 1-acetyl-4-methyl-2,5-piperazinedione was obtained in good yield from reaction with methyl iodide. The acetyl group was readily removed by treatment with isopropylamine, and the resulting 1-methyl-2,5-piperazinedione was identical with that obtained by the procedure of Chase (6). This sequence of reactions, I, indicated that the method of Chase gave a cyclic product rather than an open-chain peptide. Some doubt about the cyclization existed because of the ease of hydrolysis of 2,5-piperazinediones to peptides (9) as well as the similarity in melting points between 2,5-piperazinediones and their corresponding dipeptides in some cases.

A method resembling the cyclization procedure of Chase was found to be suitable for the preparation of 1-alkyl-2,5-piperazinediones and more convenient than the use of the sodium salt of 1-acetyl-2,5-piperazinedione. This method was based on the observation of Fisher and Fourneau (10) that ethyl glycylglycinate is readily cyclized in alcoholic ammonia to 2,5-piperazinedione. Accordingly, ethyl glycylglycinate was alkylated using methyl iodide, and the resulting N-methyl derivative was treated with dry ammonia to give the corresponding amide rather than the expected ring closure. Direct alkylation of glycylglycine was not attempted because of the insolubility of this compound in the common organic solvents. It was also necessary to neutralize the glycylglycine hydrochloride and convert it to anhydrous glycylglycine in order to isolate ethyl glycylglycine hydrochloride, which is soluble in the presence of water. In addition, even traces of moisture prevent the crystallization of ethyl glycylglycine hydrochloride. Cyclization took place when the amide was refluxed in ethylene glycol. Several 1-alkyl-2,5-piperazinediones were prepared either by this method or that of Chase (6), illustrated by the preparation of 1-ethyl-2,5-piperazinedione, and their physical properties are listed in Table II. The corresponding properties of the intermediate N-alkylglycylglycines appear in Table I.



I

Pharmacological Results.—Tests carried out by Dr. Howard J. Jenkins of the department of Biological Sciences on 1-methyl-2,5-piperazinedione showed this compound to have marked antispasmodic action on the rabbit ileum. Further pharmacological determinations will be reported.

EXPERIMENTAL[1]

Melting points were taken on a Fisher-Johns block and are uncorrected.

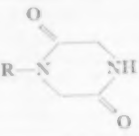
1-Myristoyl- and 1,4-Dimyristoyl-2,5-piperazinedione.—To 90 cc. of dimethylformamide was added 5.7 Gm. (0.05 mole) of 2,5-piperazinedione, prepared by the procedure of Sannié (11), and the mixture was heated at 90° until most of the 2,5-piperazinedione was dissolved. Myristoyl chloride

[1] Analyses were done by Weiler and Strauss, Oxford, Englnd, or by Carol K. Fitz, Needham Mass.

TABLE I.—N-ALKYLGLYCYLGLYCINES: $RNHCH_2CONHCH_2CO_2H$

R	M. P., °C.	Purification Solvent	Yield, %	Formula	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
C_2H_5	200–202	95% Ethanol	79	$C_6H_{12}N_2O_3 \cdot H_2O$	40.44	40.88	7.92	8.13
i-C_3H_7	213–215	95% Ethanol	72	$C_7H_{14}N_2O_3$	48.26	48.53	8.10	7.82
cyclo-C_6H_{11}	211–213	95% Ethanol	27	$C_{10}H_{18}N_2O_3$	56.05	55.84	8.46	8.41
$C_6H_5CH_2$	208–210	Abs. ethanol	21	$C_{11}H_{14}N_2O_3$	59.44	60.02	6.34	5.97

TABLE II.—1-ALKYL-2,5-PIPERAZINEDIONES:



R	M. P., °C.	Purification Solvent	Yield, %	Formula	Analyses: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
C_2H_5	172–173	Abs. ethanol	48	$C_6H_{10}N_2O_2$	50.69	50.60	7.09	7.10
i-C_3H_7	198–200	95% Ethanol	38	$C_7H_{12}N_2O_2$	53.83	53.70	7.74	7.60
cyclo-C_6H_{11}	218–219	95% Ethanol	25	$C_{10}H_{16}N_2O_2$	61.21	60.70	8.22	8.10
$C_6H_5CH_2$	218–220	95% Ethanol	50	$C_{11}H_{12}N_2O_2$	64.72	64.70	5.93	6.00

(10.9 Gm., 0.05 mole, Eastman Organic Chemicals) was then added, and heating was continued for ten minutes with stirring. The hot reaction mixture was filtered and about 0.5 Gm. of piperazinedione was recovered. The filtrate was placed in the refrigerator and after two hours was filtered. A yellow-green solid was obtained which was extracted with a total of 40 cc. of hot benzene. About 0.5 Gm. of 2,5-piperazinedione was obtained from the benzene-insoluble material. The hot benzene solution was decolorized with charcoal and concentrated, and 1.5 Gm. (9%) of 1-myristoyl-2,5-piperazinedione was obtained after recrystallization from acetone, m. p. 145–146°.

Anal.—Calcd. for $C_{18}H_{32}N_2O_3$: C, 66.62; H, 9.94. Found: C, 66.56; H, 9.83.

The filtrate from the reaction mixture was evaporated to dryness *in vacuo*, and the resulting solid was extracted with hot acetone. The acetone solution was decolorized with charcoal and allowed to evaporate. Recrystallization of the resulting solid from ether gave 3.6 Gm. (13%) of 1,4-dimyristoyl-2,5-piperazinedione which melted at 34–36°. This compound is hygroscopic.

Anal.—Calcd. for $C_{32}H_{58}N_2O_4$: C, 71.85; H, 10.93. Found: C, 72.02; H, 12.01.

Reaction of 1-Myristoyl-2,5-piperazinedione with Methyl Iodide.—A mixture of 2.4 Gm. (0.007 mole) of 1-myristoyl-2,5-piperazinedione, 75 cc. of anhydrous benzene, and 1.1 Gm. (0.007 mole) of methyl iodide was refluxed at 80° for four hours. The solution was cooled and 0.4 Gm. of 2,5-piperazinedione was recovered. The remaining solution was evaporated *in vacuo* to a volume of about 5 cc., and 1.4 Gm. of 1,4-dimyristoyl-2,5-piperazinedione was obtained, m. p. 36–38°.

Reaction of 1-Myristoyl-2,5-piperazinedione with Diethyl Sulfate.—A mixture of 30 cc. of benzene, 30 cc. of 2% aqueous sodium hydroxide solution, 0.3 Gm. (0.001 mole) of 1-myristoyl-2,5-piperazinedione, and 0.07 Gm. (0.0005 mole) of diethyl sulfate was refluxed gently for two hours. A few drops of *n*-octanol prevented foaming. After cooling to room temperature the mixture was filtered. The resulting solid was washed with hot water and hot benzene and then extracted with hot acetone to remove unreacted 1-myristoyl-2,5-piperazinedione. The remaining solid was recrystallized from 95% ethanol, and approximately 0.15 Gm. of crystals were obtained which melted at 192–194°. Elemental analysis indicated the product to be 1,4-diethyl-2,5-piperazinedione; a reported m. p. for this compound is 129° (12).

Anal.—Calcd. for $C_8H_{14}N_2O_2$: C, 56.51; H, 8.32. Found: C, 56.50; H, 9.01.

Reaction of 1-Myristoyl-2,5-piperazinedione with Benzenesulfonyl Chloride.—A mixture of 1.25 Gm. (0.004 mole) of 1-myristoyl-2,5-piperazinedione, 0.68 Gm. (0.004 mole) of benzenesulfonyl chloride, and 75 cc. of water (with a few drops of sodium hydroxide solution to bring the pH to 8) was shaken for two hours. Additional alkali was added during this period to maintain a pH of 8. The mixture was then filtered, and unreacted 1-myristoyl-2,5-piperazinedione was recovered. The filtrate was evaporated to dryness *in vacuo*, and the resulting solid was extracted with hot absolute ethanol in a Soxhlet apparatus for twenty-four hours. The extract was concentrated to about 20 cc., and 0.2 Gm. of white, crystalline material was isolated; m. p. 295–300° (with decomposition). Elemental analysis indicated it to be 1,4-dibenzylenesulfonyl-2,5-piperazinedione.

Anal.—Calcd. for $C_{16}H_{14}N_2O_6S_2$: S, 16.24. Found: S, 15.98.

Preparation of 1-Methyl-2,5-piperazinedione from 1-Acetyl-2,5-piperazinedione.—Fifty cubic centimeters of absolute methanol was treated with 0.6 Gm. (0.026 mole) of sodium, and when solution was complete, 4 Gm. (0.026 mole) of 1-acetyl-2,5-piperazinedione (7) was added. The reaction mixture was refluxed for one minute on a water bath, 11.5 Gm. (0.08 mole) of methyl iodide was added, and the mixture was refluxed for five hours. The solvent was then removed *in vacuo* and a thick syrup was obtained which was taken up in 35 cc. of anhydrous chloroform. Addition of 2 cc. of isopropylamine gave a precipitate of sodium io-

dide which was filtered. Ether, 20 cc., was added to the filtrate, which was then allowed to stand at room temperature for three days with occasional shaking. The resulting solid was isolated, washed with warm ether, and recrystallized from 2-propanol. About 1.8 Gm. (55%) of product was obtained which melted at 142–143°, which agrees with the reported value for 1-methyl-2,5-piperazinedione (6).

Preparation of 1-Methyl-2,5-piperazinedione from Glycylglycine.—*Glycylglycine.*—Twenty grams (0.11 mole) of glycylglycine hydrochloride monohydrate, prepared by the procedure of Fischer and Fourneau (10), in 40 cc. of water was treated with 10 Gm. (0.11 mole) of sodium bicarbonate. The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in a minimum amount of hot water. The solution was cooled and 50 cc. of acetone was added. After the solution was stirred vigorously for several minutes, 20 cc. of absolute ethanol was added, and a solid separated after fifteen minutes more of stirring. The product was filtered and washed with hot 95% ethanol. A yield of 13 Gm. was obtained; the m. p. was 260–261°, with decomposition, which agrees with the reported value for glycylglycine (10).

Ethyl Glycylglycinate Hydrochloride.—Dry hydrogen chloride was bubbled into an ice-cooled mixture of glycylglycine (12.0 Gm., 0.09 mole) and 250 cc. of absolute ethanol for one hour. The mixture was refluxed on a water bath for five minutes and filtered. After being cooled, the filtrate yielded 9.5 Gm. of product which melted at 181–182°, which agrees with the reported value (10).

Ethyl Glycylglycinate.—The procedure of Fischer and Fourneau (10) was used. From the previous product was obtained 4.5 Gm. of ethyl glycylglycinate which melted at 89–90°, the reported value (10).

1-Methyl-2,5-piperazinedione.—A solution of 4.0 Gm. (0.025 mole) of ethyl glycylglycinate, 30 cc. of anhydrous chloroform, and 3.6 Gm. (0.025 mole) of methyl iodide was refluxed for thirty minutes. The reaction mixture was filtered, and about 0.25 Gm. of 2,5-piperazinedione was obtained. The filtrate was evaporated *in vacuo* to a thick syrup which was taken up in 40 cc. of warm isopropyl alcohol. Dry ammonia was then passed into the ice-cooled mixture for one hour. The mixture was warmed on a water bath for ten minutes, cooled, and resaturated with dry ammonia. After standing at room temperature overnight, the mixture was filtered, and the residue was washed free of iodide ion with hot 95% ethanol. About 1.4 Gm. of compound was obtained which decomposed at 280°.

This compound was refluxed in ethylene glycol for one hour, and ammonia was liberated. After removal of the solvent *in vacuo*, crystals were obtained on cooling the resulting syrup. The product was recrystallized from isopropyl alcohol and 0.6 Gm. (19%) was obtained. The m. p. was 142–143°, the value reported for 1-methyl-2,5-piperazinedione (6).

N-Ethylglycylglycine Monohydrate.—A solution of 5.0 Gm. (0.032 mole) of chloroacetylglycine, prepared by the procedure of Levene, *et al.* (13), and 30 cc. of water was placed in an ice bath and treated slowly with 14 cc. (0.22 mole) of 70% aqueous ethylamine solution with constant shaking. The solution was then allowed to stand for two days at room temperature, and was evaporated *in vacuo* to a thick syrup. The syrup was heated on a water bath with 100 cc. of absolute ethanol, and crystallization began within fifteen minutes. After the mixture was cooled, 4.5 Gm. of product was obtained which was washed with hot 95% ethanol; m. p. 200–202°.

Anal.—Calcd. for $C_6H_{12}N_2O_3 \cdot H_2O$: C, 40.44; H, 7.92. Found: C, 40.88; H, 8.13.

1-Ethyl-2,5-piperazinedione.—A mixture of 4.0 Gm. (0.022 mole) of N-ethylglycylglycine monohydrate and 30 cc. of ethylene glycol was refluxed for thirty minutes. The mixture was evaporated *in vacuo* to a thick syrup which solidified on cooling. The residue was taken up in 75 cc. of hot absolute ethanol and decolorized with charcoal. The solution was then concentrated to approximately half its original volume and cooled. A yield of 1.5 Gm. of 1-ethyl-2,5-piperazinedione was obtained which melted at 172–173°.

Anal.—Calcd. for $C_6H_{10}N_2O_2$: C, 50.69; H, 7.09. Found: C, 50.6; H, 7.1.

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Isotonic Solutions XI*

The Permeability of Red Corpuscles to Various Sympathomimetic Amine Salts and Phenothiazine Derivatives

By EDWARD P. WINTERS† and WILLIAM J. HUSA

The effects of various sympathomimetic amine salts and phenothiazine derivatives in preventing hemolysis of human erythrocytes were studied. In some instances rabbit erythrocytes were used. The results indicated that in solutions of increasing concentrations of sympathomimetic amine salts, hemolysis initially decreased, then abruptly increased, followed by a second greater decrease and increase. In the presence of 0.6 per cent sodium chloride there was no hemolysis until a certain concentration of sympathomimetic amine salt was reached, which closely coincided with the concentration of the same drug that resulted in the final increase in hemolysis when sodium chloride was not present. Freezing point depressions and pH values of the compounds employed in the hemolytic method were determined. The compounds were tested for reactions with human serum and oxyhemoglobin. The phenothiazine derivatives tested were found to be unsuited for investigation by the hemolytic method.

PREVIOUS INVESTIGATORS (1–8) have emphasized the importance of utilizing the hemolytic method as a means of determining whether or not a solution calculated to be isotonic with the blood from physicochemical data is truly isotonic in the biological sense. It was noted that the osmotic pressure of a solute on the erythrocyte membrane depended not only on the number of solute particles in solution, but also upon whether or not the cell membrane was penetrated by these particles.

In the present investigation, different concentrations of various sympathomimetic amine salts and phenothiazine derivatives were tested in an attempt to evaluate their effect on red blood cells with respect to the prevention of hemolysis. The presence of 0.6 per cent sodium chloride gave indication of the effect of the smpathomimetic amine compounds upon erythrocytes when the sodium chloride concentration, in itself, was sufficient to prevent osmotic hemolysis.

EXPERIMENTAL

Collection of Blood.—The method of obtaining blood in the present investigation was the same as previously described by Husa and co-workers (2, 3). The blood was drawn mainly from the veins of the arms of a thirty-two year old white male and occasionally from white volunteers.

Preparation of Solutions.—The solutions utilized in the present investigation were prepared in the same manner as previously described by other workers (2, 3).

Quantitative Determination of Per Cent Hemolysis.—Determinations of hemolytic i values were made in the same manner as previously described by Grosicki and Husa (2) and Easterly and Husa (3).

Determination of pH Values.—A Fisher titrimeter, No. 9-311A, equipped with calomel and glass electrodes, was utilized in all determinations of pH values.

Determination of Precipitation on Mixing Compounds with Oxyhemoglobin.—One per cent solutions of each compound were separately mixed with equal portions of oxyhemoglobin solutions prepared by laking human erythrocytes which had been previously washed in saline solution. The mixtures of the experimental compounds and oxyhemoglobin were allowed to stand in a water bath for forty-five minutes at 37°, centrifuged for three minutes at approximately 2,000 r. p. m., and observed for evidence of precipitation. Any precipitate would result in colorimetric readings lower than those which would ordinarily be obtained from the amount of oxyhemoglobin actually released from the erythrocytes.

Determination of Precipitation on Mixing Compounds with Serum.—Serum was separated from human erythrocytes by centrifugation as above and added in like portions to 1% solutions of each compound. The mixtures were observed for forty-five minutes, during which time they were in a water bath at 37°.

Determination of Freezing Point Depression.—The apparatus used to determine the freezing point depressions, unless otherwise indicated, was that of Bartley (9) as modified by Husa and Adams (1). The freezing point depressions of various concentrations of amphetamine sulfate solutions were determined since results with this compound exhibited great fluctuations in the degree of hemolysis.

Chemicals.—The chemicals employed in the present investigation were generously donated by their manufacturers: amphetamine sulfate, dextroamphetamine sulfate, hydroxyamphetamine hydrobromide, chlorpromazine hydrochloride, prochlorperazine ethanedisulfonate, trifluoperazine dihydrochloride, and trimeprazine tartrate by Smith

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Kline and French Laboratories; phenmetrazine hydrochloride by Geigy Pharmaceutical Co.; methoxyphenamine hydrochloride by Upjohn Co.; mephentermine sulfate by Wyeth Laboratories.

RESULTS

Results of Hemolytic Determinations.—The sympathomimetic amine salts employed did not give the usual sigmoid curve (see Fig. 1) commonly obtained by this method with other compounds. Hemolysis in the presence of the sympathomimetic amine salts generally approached 100% in the lower concentrations, decreased as the drug concentration increased, then exhibited an abrupt increase in hemolysis again, only to be followed by a second decrease, which generally assumed the approximate proportions of a sigmoid curve (see Figs. 2, 4, 5, 6, 7). This second decrease in hemolysis was usually followed by a sharp increase. Each hemolysis curve shown was obtained from results with one blood sample and is representative of other curves of the same compound in different blood samples.

Results with dextro-amphetamine sulfate in both human and rabbit blood were very similar to those obtained using amphetamine sulfate in human blood.

Peculiar changes in hemolysis have been found with other compounds to a lesser degree by previous investigators (10, 11, 12) and hemolytic i values were calculated either by using only that part of the curve that was sigmoid and covered the greatest range of hemolysis, or by averaging the concentrations of the compound where the curve crossed the points of 25, 50, and 75% hemolysis more than once. In the present investigation, the former procedure was performed when possible. These hemolytic i values are compared in Table I with values of i obtained from the freezing point depressions of some of the compounds.

In the presence of 0.6% sodium chloride the concentrations of the dextro-amphetamine sulfate and hydroxyamphetamine hydrobromide solutions employed exhibited no significant hemolysis. It is important to note that with the other compounds tested, no significant hemolysis occurred in the presence of 0.6% sodium chloride until a certain concentration was reached (see Figs. 3, 5, 6, 7). Hemolysis then increased rapidly, frequently to readings on the colorimeter indicating more than 100% hemolysis, which would present the probability that some reaction had occurred between the compound and the blood components, resulting in a

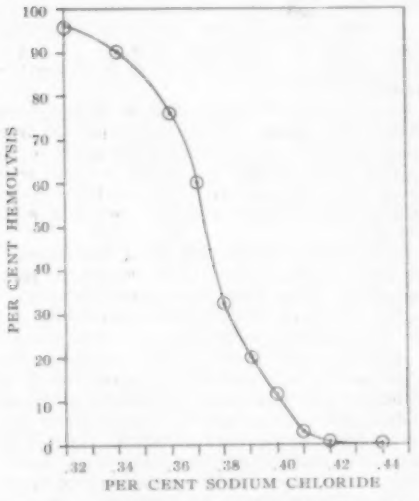

Fig. 1.—Typical curve resulting from hemolysis of human erythrocytes in sodium chloride solutions of various concentrations.

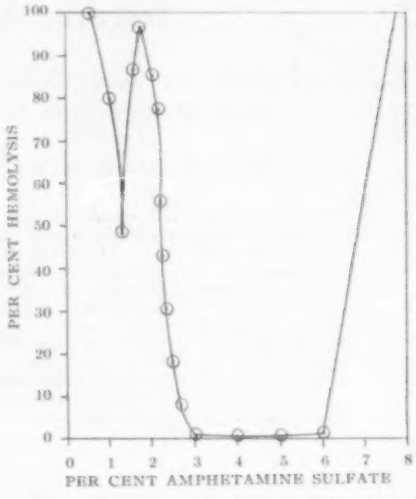

Fig. 2.—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations.

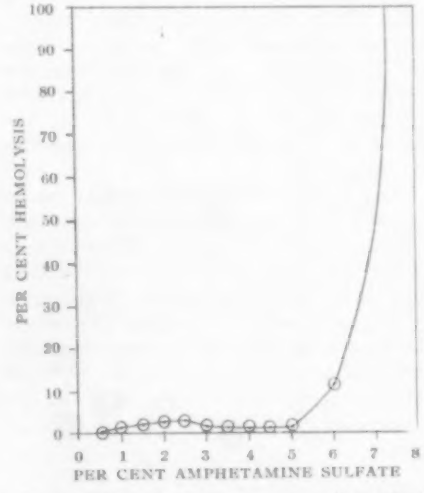

Fig. 3.—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations in the presence of 0.6% sodium chloride.

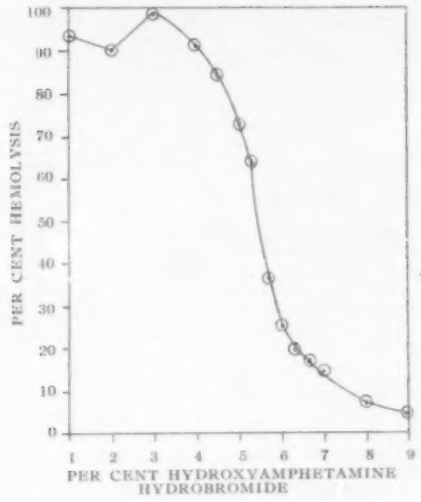

Fig. 4.—Hemolysis of human erythrocytes in hydroxyamphetamine hydrobromide solutions of various concentrations.

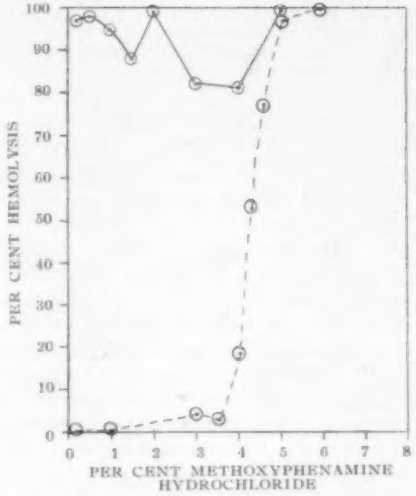

Fig. 6.—Hemolysis of human erythrocytes in various concentrations of methoxyphenamine hydrochloride in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

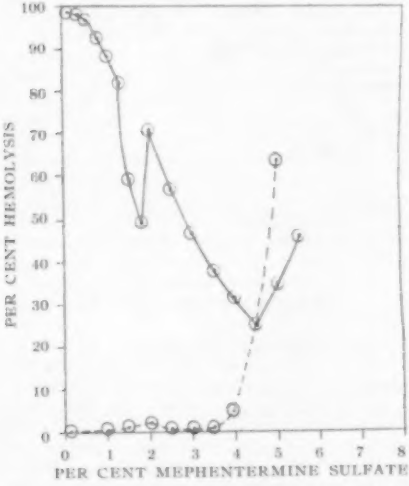

Fig. 5.—Hemolysis of human erythrocytes in various concentrations of mephentermine sulfate in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

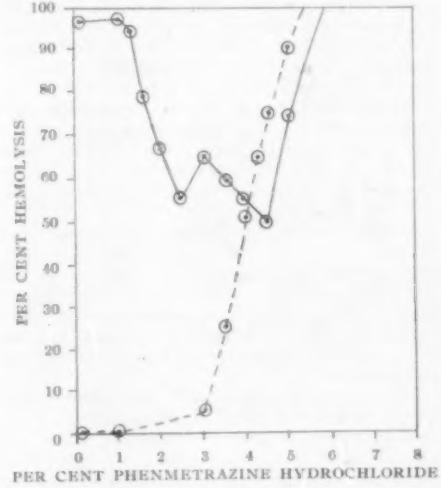

Fig. 7.—Hemolysis of human erythrocytes in various concentrations of phenmetrazine hydrochloride in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

darkening of the mixture. This same concentration closely coincided with the final upward swing in the hemolysis curves of the same compounds without sodium chloride.

Results of pH Determinations.—For the compounds subjected to the hemolytic method, the concentrations chosen for pH determinations were those at which an abrupt change in the degree of hemolysis had occurred. Since amphetamine sulfate demonstrated quite wide variations in the percentage of hemolysis with small changes in drug concentration (see Fig. 2), a more complete series of pH values was determined for this compound alone and in the presence of 0.6% sodium chloride. Phenothiazine derivatives were used in concentrations of 1% for the determination of the pH of these compounds. Some representative pH values obtained are listed in Table II.

The pH values of the sympathomimetic amine salt solutions employed ranged from 4.18 to 7.22 before the addition of human blood and 5.48 to 7.30 after

TABLE I.—COMPARISON OF *i* VALUES OBTAINED FROM FREEZING POINT DEPRESSION DATA WITH THOSE OBTAINED FROM HEMOLYTIC RESULTS[a]

Compound	Species	*i* Values—Freezing Point[b]	*i* Values—Hemolytic[c]
Amphetamine sulfate	Human	2.40	1.97
Dextro-amphetamine sulfate[d]	Human	2.40	2.03
Dextro-amphetamine sulfate	Rabbit	2.40	1.57
Hydroxyamphetamine hydrobromide	Human	2.13	0.54
Hydroxyamphetamine hydrobromide	Rabbit	2.13	0.50

[a] Unless otherwise indicated, all hemolytic values represent an average of two blood samples.
[b] Values of *i* determined from the freezing point depression of 1% solutions of the compounds (data obtained from manufacturer).
[c] Average of *i* values calculated at concentrations causing 25, 50, and 75% hemolysis.
[d] Average of three blood samples used to determine hemolytic *i* value.

the addition of human blood, thus being in the range in which pH has the least effect on hemolysis.

Of the phenothiazine derivatives used prochlorperazine ethanedisulfonate and trifluoperazine dihydrochloride had pH values of less than 3.0 in concentrations of 1%. This, in itself, would exclude the use of these two compounds in the hemolytic method due to the fact that brown colored hematin is formed from oxyhemoglobin in the presence of solutions having low pH values (13).

Results of Tests on Mixing Compounds with Oxyhemoglobin.—No precipitation was observed in any of the tubes containing 1% solutions of the compounds under investigation. Solutions of the phenothiazine derivatives turned brown upon admixture with laked erythrocytes.

Results of Tests on Mixing Compounds with Serum.—Upon addition of human serum to solutions of the compounds under investigation, an immediate cloudiness was observed in every instance. This milk-white cloud disappeared upon shaking in tubes containing sympathomimetic amine salts but remained in the tubes containing the phenothiazine derivatives.

Tests were also conducted in which defibrinated human blood was added to 1% solutions of the phenothiazine derivatives employed. When 0.1 ml. of human blood was added to 10 ml. of the solutions, the cells appeared to disperse into tiny red fragments. A gray precipitate soon appeared when the tubes were placed in a water bath at 37° and the solutions turned brown. The solution of trimeprazine tartrate contained more precipitate than the other phenothiazine derivative solutions. The precipitate which had formed in the trifluoperazine dihydrochloride solution disappeared upon agitation.

Due to the precipitates formed and the color changes of the solutions, the phenothiazine derivatives employed in this investigation could not be tested by the usual hemolytic method.

Results of Freezing Point Depression Determinations.—The freezing point depressions of the amphetamine sulfate solutions tested failed to show cause for the unusual behavior of this compound when subjected to the hemolytic method.

TABLE II.—pH VALUES FOR VARIOUS CONCENTRATIONS OF AQUEOUS EXPERIMENTAL COMPOUND SOLUTIONS BEFORE AND AFTER THE ADDITION OF HUMAN BLOOD[a]

Compound	Per Cent	Initial pH	pH, 45 min. After the Addition of Blood[b]
Blank (triple distilled water)	...	6.18	7.70
Amphetamine sulfate[c]	0.50	6.67	7.19
Amphetamine sulfate[c]	1.00	6.20	7.04
Amphetamine sulfate[c]	1.30	6.06	7.00
Amphetamine sulfate[c]	1.70	6.12	6.92
Amphetamine sulfate[c]	2.40	6.01	6.88
Dextro-amphetamine sulfate[c]	0.50	6.38	7.30
Dextro-amphetamine sulfate[c]	1.25	5.68	6.85
Hydroxyamphetamine hydrobromide[c]	2.00	5.63	6.67
Mephentermine sulfate[c]	1.80	4.59	6.99
Mephentermine sulfate[c]	2.00	4.50	6.95
Methoxyphenamine hydrochloride[c]	2.00	6.10	7.00
Methoxyphenamine hydrochloride[c]	3.00	6.05	6.99
Phenmetrazine hydrochloride[c]	1.00	4.82	5.95
Phenmetrazine hydrochloride[c]	2.00	4.60	5.80
Chlorpromazine hydrochloride[d]	1.00	5.60	5.65
Prochlorperazine ethanedisulfonate[d]	1.00	2.70	2.80
Trifluoperazine dihydrochloride[d]	1.00	2.70	2.88
Trimeprazine tartrate[d]	1.00	5.70	6.00

[a] A 0.10-ml. quantity of blood was added to 10 ml. of solution.
[b] The pH of the blood initially was 7.92, and was 7.82 after forty-five minutes.
[c] Percentages chosen due to sharp change in degree of hemolysis at these concentrations.
[d] Solutions of these compounds turned brown upon addition of blood.

DISCUSSION OF RESULTS

Factors influencing the degree of hemolysis of erythrocytes in various solutions are numerous. Hemolysis due to hypotonicity would be lessened by the simple expedient of adding more solute to the solution, thereby establishing a more nearly equal osmotic pressure between the solution and the cellular contents. A loss of electrolytes from within the cell into the external fluid would result in abnormally high hemolytic *i* values. The penetration into the erythrocyte by a divalent ion, such as the sulfate ion, would result in the release of two chloride ions from within the cell into the surrounding fluid in order to maintain electrical equilibrium, thus decreasing the number of particles within the cell. Slightly low *i* values may be indicative of incomplete dissociation, association, or micellular formation by the compound.

The hemolytic activity of many surface-active lysins has been attributed to the collapse of a cholesterol-phospholipid-lipoprotein complex in the erythrocyte surface (14). Due to the apparent surface activity of the compounds employed in the present investigation, this could possibly be a factor in some of the hemolytic results obtained. Indeed, any

form of membrane destruction, other than that due to osmotic phenomena, would cause results different from those expected from purely colligative effects.

It has been shown (15) that amine hydrochlorides may form micellular aggregates in solution with the result that their solutions have less osmotic effect than would be expected from the concentration used.

Variations in degrees of hemolysis can be partially explained not only on the basis of variations in colligative properties and membrane destruction, but also by variable reactions to the same compound by erythrocytes of the same blood sample. Love (16) and Hutchinson and Bean (17) investigated the per cent hemolysis-time curves for sodium alkyl sulfate and found that the curves were not the simple sigmoid type given by saponins, butanol, or streptolysin. The curves they obtained showed an initial rapid rate of hemolysis which decreased after a few minutes to zero, then slowly increased and continued in a sigmoid manner. Love (16) suggested that perhaps sodium dodecyl sulfate had both a protective and destructive effect. Protected cells evidently hemolyzed at a slower rate than normal cells.

Ponder (18) stated that cells of the same population would not necessarily react in a like manner to a given concentration of lysin. Differences in membrane composition or shape would result in different degrees of hemolysis at a given time from two samples of like concentrations of lysin.

It was further suggested by Ponder (19) that there may be two cell components involved in the resistance of an erythrocyte to hemolysis. The first component appears to be concerned with the mechanical rigidity and maintenance of the special shape of the cell and the other concerned with the prevention of the outward diffusion of pigment. The effects which the lysin produces on the first component are nearly always apparent before those which it produces on the second. It was theorized that some lysins affect the first component much more rapidly than they do the second, whereas others may produce their effects on the two components more nearly simultaneously.

SUMMARY

1. Increasing concentrations of sympathomimetic amine salts resulted in rather unusual hemolysis curves.

2. The sympathomimetic amine salts did not cause hemolysis in the presence of 0.6 per cent sodium chloride until a certain concentration was reached which closely coincided with the concentration of the salt alone, which resulted in the final sharp increase in hemolysis.

3. Both the *d* and *dl* forms of amphetamine sulfate gave results strongly indicative of a similar action on erythrocytes by the two compounds.

4. Determinations of the pH values before and after the addition of blood to various solutions of the sympathomimetic amine compounds indicated the acceptability of these compounds for testing by the hemolytic method.

5. No precipitation or color change occurred when solutions of laked erythrocytes and blood serum were mixed with 1 per cent concentrations of the sympathomimetic amine salts.

6. The phenothiazine derivatives employed could not be tested by the hemolytic method due to their reaction with blood.

7. The determination of the freezing point depression of various concentrations of amphetamine sulfate solutions negated the possibility of micelle formation of this compound in the concentrations used as being responsible for changes in its hemolysis curve.

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Canine Blood Sugar and Lactic Acid Responses to Adrenergic Amines after Ganglion Block*

By R. S. McCUTCHEON

Since preliminary studies indicated that a ganglionic block before infusions of some adrenergic amines resulted in an increased production of blood lactic acid and blood sugar, it was postulated that this might be due to an interruption of Cori's cycle. This experiment shows that there is a significant increase in lactic acid produced by two of the drugs after blocking and an indication that two others also have this effect. The results on blood sugar were not significant. Evidence is presented here that the conversion of lactic acid to glycogen in the liver is blocked or inhibited by use of the ganglion blocking agent, mecamylamine, accounting for an accumulation of blood lactic acid under the conditions of the experiment.

SEVERAL investigators (1) have indicated that production of lactic acid is the mechanism by which smooth muscle is relaxed, and that the formation of lactic acid is the first function of epinephrine, not the release of glucose (2). Other investigators (3) have indicated that the increased lactic acid is the result of glucose metabolism (4, 5). Most of this work has been done on epinephrine alone and in only a few instances has the work been related to the effect of ganglion blocking agents (6, 7, 8). Preliminary work in our laboratory has indicated that the increase in blood lactic acid produced by injections of adrenergic amines is greater after a ganglionic block than without it. The potentiation of the lactic acid response under these conditions has led us to postulate that it may be due to a block or an inhibition of the synthesis of lactic acid to glycogen by the liver in Cori's cycle (9). If at the same time the production of lactic acid by muscle is increased by the amine there must be an accumulation of lactic acid. The present study was undertaken to further investigate this problem in relation to epinephrine and other adrenergic amines before and after ganglionic block, and as a preliminary to a similar study in relation to these effects after adrenergic block.

Thus, a comparative study was carried out in the anesthetized dog of the blood sugar and lactic acid effects after ganglionic block, using epinephrine, isoproterenol, ethylnorepinephrine, and the N-isopropyl derivative of ethylnorepinephrine (Win-3046).

EXPERIMENTAL

Method.—Mongrel dogs of either sex weighing 10 to 15 Kg. were anesthetized with pentobarbital sodium, 35 mg./Kg., intraperitoneally. No morphine nor other preanesthetic agent was used at any time. Blood sugar was determined according to the method of Nelson (10); lactic acid by the method of Barker and Summerson (11), after deproteinizing by the method of Folin-Wu, Van Slyke and Hawkins (12).

Constant infusions of the adrenergic drug were made into the jugular vein and samples were drawn from the saphenous vein of the hind leg. Control blood sugar and blood lactic acid were obtained before starting the infusion. Alternate dogs were given the ganglionic blocking agent, mecamylamine, 1 mg./Kg. and in some cases, 2 mg./Kg. When mecamylamine was used it preceded the infusion of the adrenergic agent. Control studies were also made in which mecamylamine was followed by infusions of physiological saline solution duplicating the infusions of the amines. No change in lactic acid production occurred. The amines were infused by means of a syringe actuated by a stepping relay controlled through an electronic counting device (13) or by an infusion pump manufactured by the Harvard Apparatus Co.

Infusions of increasing doses of the drugs lasting about two hours were used in order to determine degree of altered response to increasing doses.

TABLE I.—CONCENTRATION OF DRUGS AND RATE OF INFUSION*

Time Intervals, min.	Epinephrine	Ethylnorepinephrine	Win-3046	Isoproterenol
1	0.153	2.15	2.54	0.111
2	0.613	4.3	5.07	0.444
3	1.033	8.6	10.14	1.14
4	1.533	10.74	12.68	1.7
5	3.066	21.40	25.30	2.28
6	4.599	42.80	50.75	4.56
7	9.200	86.00	101.48	9.12

* Doses in mcg./Kg./min.

* Received March 15, 1960, from the Department of Pharmacology, School of Pharmacy, Oregon State College, Corvallis.

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The author wishes to express appreciation to Dr. R. P. Ahlquist for his counsel on this project, and to David A. McClure for his help with the chemical analysis.

Drugs were kindly furnished as follows: mecamylamine (Inversine) by Merck, Sharp and Dohme, and the amines used [ethylnorepinephrine (Butanephrine), isoproterenol (Isuprel), and Win-3046] by Sterling-Winthrop Research Institute.

TABLE II.—BLOOD SUGAR AND BLOOD LACTIC ACID INCREASES BEFORE AND AFTER GANGLIONIC BLOCK

Drug	Average Increase without Block, mg. %				Average Increase after Block, mg. %				Differences, mg. %	
	Blood Sugar	No. Animals Used	Lactic Acid	No. Animals Used	Blood Sugar	No. Animals Used	Lactic Acid	No. Animals Used	Blood Sugar	Lactic Acid
Epinephrine	167	12	23	12	133	5	24	8	−35	+1
Ethylnorepinephrine	140	4	23	5	140	3	27	4	+0.4	+4
Win-3046	180	3	33	4	185	3	43	5	+5	+10
Isoproterenol	143	4	27	4	128	7	42	7	−16	+14

It was also of interest to determine whether or not the larger concentrations of drug would themselves have an inhibiting effect. Blood samples were analyzed at twenty-minute intervals during the infusion in order to determine what changes were taking place with increasing doses of the amine. Since in every case the increases in blood sugar and blood lactic acid reflected the increasing concentration of the infusion, the figures used in the reported results were the averages of the values obtained at the end of the infusion.

The solutions of the amines used contained 0.1% each of chlorobutanol and sodium bisulfite as preservative. The concentration of drug and rate of infusion are shown in Table I. Note that the dosage of the drugs has been adjusted at each level to give approximately equal effects in terms of known responses.

RESULTS

The increases in blood sugar and blood lactic acid produced by the infusions before and after ganglion block were obtained by taking the differences between the control (value before injection of any drug) and the final value for each experiment. These results were averaged for each series of like experiments. Table II shows the figures, in mg. %, obtained in this way.

Statistically, this is a 2 × 4 factorial experiment with disproportional numbers of observations in the subclasses. Without blocking and after blocking constitute one factor, and the four amines constitute another. The number of animals in the eight subclasses for lactic acid and blood sugar are shown in Table II.

TABLE III.—ANALYSIS OF VARIANCE FOR LACTIC ACID

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Drug	1,675.39	3	558.46	4.88[a]
Blocking	563.32	1	563.32	4.92[b]
Interaction	339.45	3	113.15	0.99
Error	4,689.98	41	114.39	..

[a] Significant at 1% level. [b] Significant at 5% level.

Lactic Acid.—The analysis of variance of lactic acid data is shown in Table III. The four sympathomimetic amines in the absence of a blockin g agent produced statistically significant increases in the levels of lactic acid.

In the presence of the ganglion blocking agent, all four sympathomimetic amines led to statistically significant increases in lactic acid levels. In the case of two of the amines, Win-3046 and isoproterenol, the increase in lactic acid concentration was significantly greater with the blocking agent than without it. It can be said that the blocking agent had an effect on lactic acid levels and that in no case was the production of lactic acid blocked.

Blood Sugar.—The analysis of variance of blood sugar data is shown in Table IV. The four sympathomimetic amines in the absence of a blocking agent produced statistically significant increases in the levels of blood sugar.

In the presence of a blocking agent, all four sympathomimetic amines led to statistically significant increases in blood sugar levels. The difference between blocking and nonblocking and interaction are not significant. Examination of the data reveals that the experimental variation is large and the number of animals is small. Under these conditions, blocking is not significant.

DISCUSSION

This study has shown that blood sugar and blood lactic acid are increased by infusions of the four amines: epinephrine, ethylnorepinephrine, Win-3046, and isoproterenol. This increase is large whether the infusion is made with or without the ganglion blocking agent mecamylamine. In the case of two of the amines, Win-3046 and isoproterenol, the increases of lactic acid were significantly greater after the use of the blocking agent.

An examination of the lactic acid differences of Table II is very suggestive since these appear in the same order as that postulated for the β-stimulating effects of these four substances (14, 15).

Since the results for blood sugar are not consistent and the differences in the increases before and after block are not significant, it would seem that the greater production of lactic acid after block is not

TABLE IV.—ANALYSIS OF VARIANCE FOR BLOOD SUGAR

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Drug	9,262.58	3	3,087.53	0.824
Blocking	1,121.59	1	1,121.59	0.299
Interaction	2,323.21	3	744.40	0.207
Error	123,647.02	33	3,746.88	...

dependent on a similar change in the blood sugar. This is felt to be evidence in support of the original postulation, that the block produced must be one in Cori's cycle, preventing a rapid return of the lactic acid produced to liver glycogen.

SUMMARY

1. Infusions of the four amines, epinephrine, ethylnorepinephrine, Win-3046, and isoproterenol in dogs produce large and significant increases in both blood sugar and lactic acid with or without the blocking agent.

2. When infusions are made after a ganglionic block produced by mecamylamine, the increase of blood sugar is variable but the production of lactic acid is increased significantly by two of the amines, Win-3046 and isoproterenol, over that seen without the block.

3. If the lactic acid increases after blocking are placed in order of magnitude, we find that the four amines are thus arranged in the order of their β-stimulating properties.

4. Evidence is presented here that the conversion of lactic acid to glycogen in the liver is blocked or inhibited by the use of the ganglion blocking agent, mecamylamine, accounting for an accumulation of blood lactic acid under the conditions of the experiment.

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Evaluation and Technology of an Emollient Suppository Base*

By H. I. SILVERMAN

A requisite in the medicinal treatment of local conditions involving the anorectal area is for an emollient, nongreasy, protective application that would favor a sustained release of incorporated medicinals in addition to maintaining the mucous epithelial lining of the area in its normal hydrated condition. Utilizing Lantrol, a nonsensitizing liquid fraction of wool fat, as the major component, a new suppository base has been formulated which possesses, in addition to the foregoing attributes, a high degree of pharmaceutical elegance, adequate plastic range, and is compatible with the great majority of pharmaceuticals commonly incorporated in suppositories. Techniques utilized to determine rate of pharmaceutical release show a desirable prolonged release of medication. Sensitivity testing has also been accomplished. While the base itself is not water soluble it is dispersible in body fluids, and warm water, forming a water-in-oil emulsion that spreads smoothly over the skin forming a protective, partially occlusive, nongreasy film. No difficulties were noted in manufacture, the base sets rapidly and stabilizes quickly.

IN RECENT YEARS a great deal of consideration has been afforded suppositories from the standpoint of base formulation (1–5). Few studies, however, have been devoted to the development of a base having a therapeutic activity of its own in addition to being a vehicle for various types of medication.

The great majority of the available vehicle bases melt, disintegrate, or dissolve rapidly at body temperature. In this way medicinal agents are expected to be rapidly absorbed should systemic effects be desired. However, these bases are not especially suited for treating local conditions involving the anorectal area. The widely used fatty bases such as cacao butter and its inexpensive substitutes, the hydrogenated fats, are prone to allow leakage from the anal-

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sphincter while not holding medication *in situ* over extended periods of time. In addition, their low melting point (6) is somewhat unsuited for suppositories that are produced on a commercial scale since they produce numerous problems during their manufacture, storage, shipping, and handling. The water-soluble and emulsified bases are preferable to the oleaginous since they permit increased diffusion of medication. Ingredient release, however, may be extremely rapid so that a desirable prolonged and steady medicament release, a requisite in treating anorectal inflammations, does not occur. Suppositories formed from various synthetic gums, natural colloids, and protein materials are not especially suited since they are hydrophilic, tend to swell, and may induce the defecation reflex.

With these considerations in mind, studies were channeled toward the development of a suppository that would: (*a*) be serviceable as a vehicle for medication commonly incorporated in this dosage form; (*b*) be stable under normal storage conditions over an extended time; (*c*) involve no special precautions or procedures during manufacture and eliminate the problems of chipping, fissuring, and splitting; (*d*) not require a mold lubricant, solidify rapidly (high setting point), and eject easily, and (*e*) be nonirritating, not leak from the rectum, and able to be handled without deformation.

In addition, the base should be emollient, nongreasy, protective, and allow for a sustained release of incorporated medicinals while maintaining the mucous epithelial lining of the anorectal area in its normal hydrated condition. Since the introduction of wool fat and its derivatives into medicine many references attest to the value of this animal wax as an emollient and adhesive application (7–9). However, due to some manifestations of eczematous hypersensitivity (10) its utilization in dermatology must be with caution to avoid exciting an allergic response in sensitive individuals.

Since the sensitizing component can be demonstrated to be present in the alcohol fraction (11) removal of this constituent by fractionation or chemical alteration such as acetylation results in a hypoallergenic, medicinally and cosmetically acceptable lanolin. Lantrol[1] (12), a liquid fraction of wool grease, was chosen as the major component of this new suppository base. Utilization of a nonsensitizing lanolin for local application should have therapeutic value as wool fat has been demonstrated to enhance the mechanism for rehydration of the skin (13), to provide a protective, partially occlusive smooth film, and to be miscible with water forming a water-in-oil emulsion which, in view of its biological origin, may be somewhat analogous to human sebum (9, 10).

Other materials utilized in formulation as thickening and molding agents were the following: Myverol 18-00,[2] a chemical isolation product of hydrogenated lard, high in monoester content obtained by high vacuum molecular distillation and Wecobee base S,[3] a partially hydrogenated oxidatively stable fat which may be utilized as a substitute for cacao butter.

EXPERIMENTAL

Formulation and Physical Data.—In the screening to evolve a suitable suppository vehicle, 75 raw materials were utilized for the preparation of over 50 experimental bases. Of those studied, a base having the following composition was selected: Lantrol, 40%; Wecobee base S, 40%; Myverol 18-00, 20% by weight. The ingredients were combined by fusion at approximately 80–85° with stirring to produce a clear golden-yellow liquid. Molding was accomplished by pouring the fused base at its cloud point of 55° (50–55°) into previously chilled suppository molds. After chilling at 0° for thirty minutes the suppositories were ejected from the mold and stored at 5° for five days in order to allow the isomeric components to assume stable crystalline patterns. The suppositories were then subdivided for the stability studies. Suppositories in dual groups of twenty-five (one group wrapped in aluminum foil, the other with no protective covering) were stored in a refrigerator, at room temperature (20–25°), and on a window ledge exposed to sunlight.[4] After three months each suppository was carefully examined for any evidence of physicochemical change. None in the entire group of one hundred and fifty suppositories showed any evidence of color change, blooming, or deformation.

The softening point of the suppository base was carried out utilizing the ring and ball apparatus of the A. S. T. M. and found to fall within the range of 47–48°. A suppository slice was packed into the center of the metallic ring with the ball situated on top of the suppository segment. The apparatus was then immersed in a water bath at a temperature of 25° which was heated by a hot plate equipped with a magnetic stirrir to maintain an even temperature. The rate of temperature rise was regulated at 1° per minute. The softening point range being read when the metallic ball fell free from the metallic ring. Three separate determinations were performed on newly molded lots and on the variously stored samples after the three month aging period. In no case was there any variation from the aforementioned softening point range.

[1] Supplied by Malmstrom Chemical Corp.

[2] Supplied by Distillation Products Industries.

[3] Supplied by E. F. Drew Co.

[4] This latter group was placed in a specially constructed container, of window glass, with the environmental temperature controlled so as not to rise above 35°.

The pH of a dispersion of the base in distilled water was found to be 5.6. This was determined by mixing 5 Gm. of the base with 100 ml. of distilled water for several minutes, filtering, and reading the pH by means of a Beckman Zeromatic pH meter.

Consistency was determined with a Universal model precision penetrometer. A 15-square cm. cast of the base was utilized for the penetration. The average of five readings (corners and center) taken with the penetrometer needle was 16.08 mm.

The specific gravity of the suppository base was found to be 0.93.

Determination of Medicinal Release.—A colorimetric and a microbiological study was accomplished to ascertain the ability of the base to release incorporated medicinals.

The colorimetric method was performed simultaneously with the Lantrol base and a cacao butter base. Molded and aged suppositories containing known amounts of FD&C Red No. 2 were placed in constant temperature water baths, equipped with magnetic stirrirs, set at the softening points of the bases in question. At various intervals aliquot samples were removed, filtered with sintered-glass funnels, and the concentration of dye, as released over the time period of study, determined with a Coleman Jr. spectrophotometer at 525 mμ. Using the data obtained graphs were plotted (Fig. 1) to compare the percentage release of dye from the two bases at varying time intervals. The procedure followed is similar to one originally outlined by Gross and Becker (2).

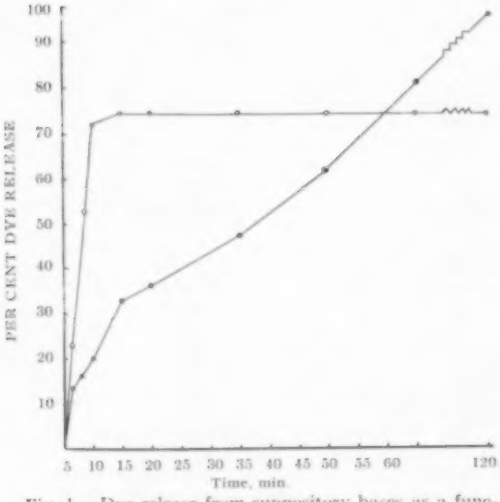

Fig. 1.—Dye release from suppository bases as a function of time. ○, Theobroma base plotted at 37°; ●, Lantrol base plotted at 47°.

Antibacterial activity in regard to the effectiveness of release of certain selected medicinals from the Lantrol base as compared to a cacao butter base (containing the same ingredients in exactly the same proportion) was determined using four test organisms, Table I. The method followed was to place a freshly sliced segment of a suppository cone containing the medicinal in question, 10 mm. in diameter and 10 mm. in thickness, upon seeded agar plates. To avoid softening and creeping at the generally accepted incubation temperature of 37° the plates were allowed to remain at room temperature (20–25°) for forty-eight hours before being read. Zones of inhibition were measured from the periphery of the suppository segment to the limit of the clear area, the size of the zone being directly related to the ability of the base to release incorporated materials.

Sensitivity Studies.—To determine any possible evidence of sensitivity to the base a modified repeat-insult patch technique was performed using a pressure type of patch application (14–17). The method used takes into account the possibility of fatiguing of the skins defense mechanism while also serving to demonstrate possible sensitization build-up over a time lapse.

An interracial group of 60 human subjects were used for the experimental study consisting of 55 males and 5 females, ages ranging from 18–30. Approximately 350–500 mg. of the test-base was applied during each twenty-four-hour testing period. Subjects were instructed to apply the test material to the underside (ventral) of the forearm, midway between the elbow and wrist. The test area covered approximately 10 sq. mm. of the subjects skin. The materials used in applying the base consisted of a felt pad, doughnut shaped, whose center opening was filled with the base. The pad and base were placed on the skin, covered with a suitable gauze pad, and firmly affixed with adhesive to the skin. Subjects were instructed to apply the patch for twenty-four hours, remove patch, wash area, and rest twenty-four hours, apply again for twenty-four hours, remove patch, wash area, and rest twenty-four hours, etc., until five patch applications

TABLE I.—ZONES OF INHIBITION

Medicament	Concn. in Suppository	Base	Test Organism: *Proteus Morganii*, mm.	*Klebsiella pneumoniae*, mm.	*Micrococcus pyogenes citreus*, mm.	*Micrococcus pyogenes albus*, mm.
Penicillin G pot.	200,000 u.	Lantrol	5	3	15	14
		Theobroma	...	...	7	4
Tetracycline	100 mg.	Lantrol	10	10	13	19
		Theobroma	10	7	10	14
Nitrofurazone	0.2%	Lantrol	5	...	3	10
		Theobroma	2	...	...	...
As in No. 37, Table III	As in No. 37, Table III	Lantrol	...	5	3	9
		Theobroma	Growth stimulated	Growth stimulated	...	...

TABLE II.—SENSITIVITY TESTS

Subject No.	Sex	Medical History[a]	Age, yr.	Conclusions
1	M	...	21	Negative
2	M	"Hay fever"	19	Negative
3	M	Severe acne	19	Slight pruritis first 24 hr. only, negative thereafter
4	M	...	20	Negative
5	M	...	19	Negative
6	M	...	18	Negative
7	M	"Hay fever"	18	Negative
8	M	...	20	Negative
9	M	...	19	Negative
10	M	...	22	Negative
11	M	...	19	Negative
12	M	...	23	Negative
13	M	...	19	Negative
14	M	"Hay fever"	19	Negative
15	M	...	19	Slight pruritis for first few minutes of first application
16	M	...	20	Negative
17	M	...	21	Negative
18	M	Acne	20	Negative
19	F	Food allergies	18	Negative
20	M	"Hay fever"	19	Negative
21	M	...	28	Negative
22	F	Asthma	26	Negative
23	F	...	18	Negative
24	M	...	19	Negative
25	M	...	21	Negative
26	M	"Hay fever"	26	Negative
27	M	"Hay fever"	30	Negative
28	F	...	29	Negative
29	M	...	21	Negative
30	M	...	27	Negative
31	M	...	20	2-mm. Wheal at end of first application only
32	M	...	19	Negative
33	M	...	20	Negative
34	M	"Summer rash"	19	Negative
35	M	...	19	Negative
36	M	Warts on legs	19	Negative
37	M	...	20	Negative
38	M	"Hay fever"	19	Negative
39	M	...	20	Negative
40	M	Severe acne	19	Negative
41	M	...	19	Negative
42	M	...	19	Negative
43	M	Food allergies	22	Negative
44	M	...	20	Negative
45	M	"Hay fever"	19	Negative
46	M	...	20	Negative
47	M	...	19	Negative
48	M	Impetigo	19	Negative
49	M	...	19	Negative
50	M	...	20	Negative
51	M	...	20	Negative
52	M	...	19	Negative
53	M	...	19	Negative
54	M	...	20	Negative
55	M	...	20	Negative
56	M	...	19	Negative
57	M	...	19	Negative
58	M	"Hay fever"	19	Negative
59	M	"Hay fever"	19	Negative
60	F	...	20	Negative

[a] "Hay fever," asthma, gastrointestinal disturbances, urticaria, serum sickness, dermatitic diseases etc.

had been completed. After a resting period of thirty days, a second series of five patch applications were completed.

Subjects were instructed to record any erythema, pruritus, tingling, eruptions, or any other abnormalities during the test periods. Table II records subject data, including medical histories, and observations on the sensitivity tests.

TABLE III.—COMPATIBILITY STUDIES

No.	Medication	Amt. per Suppos.	Flux Temp., °C.	After 3 Months Storage[a] At RT	Refrigerator
1	Aminophylline	0.5 Gm.	75	C	A
2	Aspirin	0.5 Gm.	65	A	A
3	Argyrol	0.3 Gm.	60	A	A
4	Belladonna ext.	15 mg.	60	A	A
5	Benzocaine	0.15 Gm	60	C	A
6	Bismuth subcarb	0.2 Gm.	70	A	A
7	Bismuth subgallate	0.2 Gm.	70	A	A
8	Boric acid	0.15 Gm.	60	A	A
9	Butabarbital	65 mg.	70	A	A
10	Stilbestrol	5 mg.	65	A	A
11	Ephedrine sulf.	5 mg.	65	A	A
12	Ichthamol	0.3 Gm.	60	D	D
13	Mercurochrome	0.2 Gm.	70	A	A
14	Nitrofurazone	0.2 Gm.	65	A	A
15	Pentobarb. sod.	65 mg.	70	A	A
16	Peruvian balsam	0.2 Gm.	50	D	A
17	Phenobarbital	65 mg.	70	A	A
18	Succinylsulfathiazole	0.5 Gm.	60	A	A
19	Sulfamethoxypyridazine	0.25 Gm.	65	A	A
20	Tannic acid	0.3 Gm.	70	A	A
21	Vioform	3.0%	65	A	A
22	Zinc oxide	0.2 Gm.	60	A	A
23	Chloramphenicol	100 mg.	60	A	A
24	Chlortetracycline	100 mg.	60	A	A
25	Erythromycin	100 mg.	60	A	A
26	Neomycin sulf.	10 mg.	60	A	A
27	Oxytetracycline	100 mg.	60	A	A
28	Penicillin G pot.	65 mg.	60	A	A
29	Polymyxin B sulf.	10 mg.	60	A	A
30	Streptomycin	200 mg.	60	A	A
31	Tetracycline	100 mg.	60	A	A
32	Cortisone acetate	20 mg.	65	A	A
33	Dexamethasone	1 mg.	65	A	A
34	Hydrocortisone acetate	20 mg.	65	A	A
35	Prednisone	5 mg.		A	A
36	Benzocaine	120 mg.		B	A
	Ephedrine hyd.	5 mg.			
	Vioform	15 mg.	60		
	Zinc oxide	150 mg.			
	Peruvian balsam	150 mg.			
37	Belladonna ext.	15 mg.			
	Ephedrine sulf.	3 mg.			
	Zinc oxide	100 mg.	60	A	A
	Boric acid	100 mg.			
	Bismuth subcarb.	100 mg.			
	Peruvian balsam	100 mg.			

[a] A, no change, satisfactory; B, blooming; C, color change; D, softening or deformation.

Compatibility Tests.—Several pharmaceuticals[5] which are commonly incorporated in suppository formulations were screened in the evaluation of the Lantrol base as a vehicle for medication. Materials listed (Table III) were, if in the form of a dry, free flowing powder, screened to pass through a number 200-mesh sieve prior to incorporation. Percentages selected were based on those generally found in commercial practice. Incorporation of ingredients into the base was by fusion into the melted base at a temperature found to be optimum for the material in question (Table III), and was accomplished by utilizing a hot plate equipped with a magnetic stirring device. The individual mixtures were stirred continuously while being poured into previously-chilled suppository molds. All molded suppositories were then aged for five days at 5°. At the completion of the initial cold storage those suppositories containing similar materials were divided into groups of twenty, with one group being wrapped in aluminum foil and the other having no protective covering. All molded suppositories, wrapped and unwrapped, were then placed in storage for a period of three months, both in a refrigerator and at room temperature. At the expiration of this time the suppositories were carefully examined to detect any evidence of incompatibility with the incorporated medicinals. Suppositories were checked for color changes, odor, blooming, cracking, softening, deformation, and spotting. Analytical determinations were not attempted during this portion of the investigation.

[5] The following companies supplied pharmaceuticals utilized in the compatibility investigation: American Cyanamid Co., Burroughs Wellcome & Co., Eaton Laboratories, Eli Lilly & Co., Merck Sharp & Dohme Research Laboratories, Pfizer Laboratories, Upjohn Co.

DISCUSSION AND CONCLUSIONS

In view of the fact that wool fat is generally accepted as the emollient of choice for medicinal pur-

poses it appeared feasible that it would be of utility, when properly formulated, into an emollient suppository vehicle. In addition to its value as a softening and lubricating agent it also has an affinity for the skin which differentiates it from other similarly used materials. Due to this adhesive property it should be far more suitable (7) to prolong the effects of incorporated medication than other fats or oils. Figure 1 and Table I attest to the value of the Lantrol base regarding release of incorporated material. In addition to a prolonged, sustained action, just short of the entire amount of incorporated dye was detected at the end of a two-hour testing period. While the base itself is not water soluble it is dispersible in both body fluids and warm water forming a w/o emulsion which spreads out smoothly over the skin forming a protective, partially occlusive, nongreasy film.

In comparison the cocoa butter base released the dye very quickly and, since it lacks the coherent property of lanolin, could not be expected to maintain the medication in close contact with the surrounding tissue. Actually, leakage and corresponding loss of medication may be a problem. In addition, it can be noted that there is almost a 30% greater release of dye from the Lantrol base than the theobroma base. Since stability and resistance to deformation are important from a commercial standpoint the suppositories were formulated so as to have a softening point analogous to proprietary products (18).

Also of value from a manufacturing standpoint is the high suspending power of the base for materials with a high density; settling of incorporated medicinals during the mold pouring stage is thusly reduced to a minimum.

The lack of irritation in the group of subjects employed for the sensitivity studies (Table II) illustrates that this base is nonallergenic.

In view of the relatively few abnormalities detected in the compounded lots (Table III) it may be concluded that the great majority of pharmaceuticals are compatible, save, as might be expected, resinous type products for which the base could be slightly modified. Benzocaine appears too complex with the base in view of the fact that both numbers 5 and 36 of the compounded series showed evidence of abnormalities (Table III). The color change or blooming, which occured in some samples, was detected only in the unwrapped suppositories; those enclosed in aluminum foil exhibited no evidence of color change or blooming.

In view of the accumulated data it would appear that the proposed Lantrol suppository base has a high degree of pharmaceutical elegance, excellent stability, adequate plastic range, and is compatible with the great majority of pharmaceuticals commonly incorporated in this dosage form.

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Determination of Hydrochlorothiazide in Urine*

By HERBERT SHEPPARD, THOMAS F. MOWLES, and ALBERT J. PLUMMER

Hydrochlorothiazide may be determined in urine by extracting with ethyl acetate and either reacting directly with chromotropic acid or hydrolyzing first with alkali followed by diazotization and condensation with N-(1-naphthyl)-ethylene diamine hydrochloride.

THE RECENT introduction of the new oral diuretic, hydrochlorothiazide[1] (6-chloro-7-sulfamyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide), which is several-fold more potent than the parent compound chlorothiazide, has necessitated the development of more sensitive and specific methods of analysis. Baer, *et al.* (1), recently described the use of the Bratton-Marshall reaction on diluted urine which had been first hydrolyzed with alkali. Unfortunately, this method may not satisfactorily be applied to the determination of hydrochlorothiazide. The use of much smaller doses of this diuretic does not permit one to make the dilutions called for in the Bratton-Marshall reaction. With smaller dilutions, however, interference is encountered from unknown components in the urine as observed earlier by Marshall, *et al.* (2). This interference can be corrected for by adding two to three different levels of the drug to additional aliquots of the same urine sample and using the values obtained as a standard curve. This method, however, suffers from a lack of sensitivity. The following sections describe a method of separating the drug from interfering substances and the development of a new method which is more specific in that it becomes possible to distinguish hydrochlorothiazide from chlorothiazide and conjugated sulfonamides.

MATERIALS AND METHODS

Preparation of Urine Extracts.—One milliliter of urine is diluted with 2 ml. of distilled water and extracted twice with 18 ml. of ethyl acetate. The pooled ethyl acetate extracts are evaporated to dryness *in vacuo* at 45°.

Method A.—Add 1 ml. of 5 *N* NaOH to the dried residue and place in boiling water bath for thirty minutes. Cool and dilute with 8 ml. of distilled water. Acidify with 1 ml. of concentrated HCl. Add 1 ml. of 0.1% aqueous solution of sodium nitrite, mix, and let stand four minutes. Add 1 ml. of 0.5% aqueous solution of ammonium sulfamate, mix, and let stand three minutes. Finally add 1 ml. of 0.1% aqueous solution of N(1-naphthyl)-ethylene diamine dihydrochloride and within thirty seconds to one minute read the absorbance in the Klett-Summerson colorimeter fixed with a No. 54 filter.

Method B.—To the residue from the ethyl acetate extract of the urine is added 5 ml. of acetone along the sides of the tubes. Not all of the material may dissolve so that centrifugation may be necessary. At this point 0.5 ml. of acetone is removed and evaporated to dryness. To the dried residue is added 1 ml. of 15 *M* H_2SO_4 containing 2 mg./ml. of chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid). Heat for five minutes in a boiling water bath, cool, and read color at 570 mμ.

For both methods standard curves are prepared by adding 20–100 mcg. of drug to control urine samples and carrying them through the analysis. The standard solutions are prepared by dissolving hydrochlorothiazide in acetone at a concentration of 1 mg./ml. The appropriate aliquot is placed in an empty tube, the acetone evaporated off, and the urine sample added.

RESULTS

In preliminary work using hydrochlorothiazide labeled with tritium it was observed that the ethyl acetate extracts the drug quantitatively from the urine. This effectively separates the drug from most of the interfering substances present. As observed in Table I, hydrochlorothiazide may be readily hydrolyzed in five minutes by strong acid or alkali with the degree of hydrolysis decreasing with decreasing normality. Since the use of acid hydrolysis gives a lower color intensity, alkaline hydrolysis was resorted to prior to doing the Bratton-Marshall reaction. In Fig. 1 it is observed that time as well as concentration of alkali are important factors in effecting a greater degree of hydrolysis. After

TABLE I.—EFFECT OF NORMALITY OF ACID AND ALKALI ON EXTENT OF HYDROLYSIS OF HYDROCHLOROTHIAZIDE

Reagent	Normality	Relative Degree of Hydrolysis, 5 Min.*
H_2SO_4	6.00	100
	3.00	83
	1.50	62
	0.75	42
	0.38	24
	0.19	13
NaOH	10.00	100
	5.00	85
	2.50	46
	1.25	17
	0.60	12
	0.30	7
	0.15	3

* These values are arbitrarily set at 100 and do not necessarily represent complete hydrolysis.

* Received October 12, 1959, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
A preliminary report of this work was presented at the 135th Meeting of the American Chemical Society, Boston, Mass., April 1959.

[1] Ciba's trademark for hydrochlorothiazide is Esidrix.

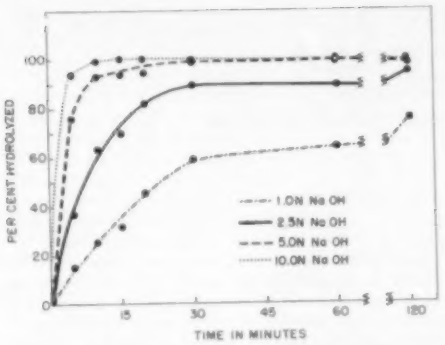

Fig. 1.—Effect of alkalinity on the degree of hydrolysis of hydrochlorothiazide. Maximum color development under these test conditions is taken as 100% hydrolysis. 200 mcg. hydrochlorothiazide in 2 ml. solution at 100°.

coupling with the N(1-naphthyl)-ethylene diamine there is a tendency for some urine samples to increase in intensity of color. This is due to the presence of interfering substances which are not completely eliminated by extraction with ethyl acetate. It is therefore advisable to read the samples within one minute, at which time color development with the drug alone is complete and remains stable for at least fifteen minutes.

The reaction with chromotropic acid is very rapid with maximum color being reached within five minutes and remaining stable for at least several hours. The use of acetone to extract the ethyl acetate residues is necessary to remove interfering substances further. Table II compares the chromotropic acid reaction with the Bratton-Marshall reaction on the urines from some dogs which had received hydrochlorothiazide orally. It is observed that the two methods give essentially identical results.

TABLE II.—COMPARISON OF CHROMOTROPIC ACID AND BRATTON-MARSHALL REACTIONS FOR HYDROCHLOROTHIAZIDE IN DOG URINE

Dog. No.	Collection Period, hr.	Hydrochlorothiazide, mcg./ml. of Urine	
		Bratton-Marshall	Chromotropic Acid
59	0–2	157	162
	2–4	68	66
	4–6	57	46
	6–7.4	72	64
	7.4–24	20	26
25	0–2	115	118
	2–4	60	54
	4–6	55	...
	6–7.4	54	52
	7.4–24	20	22

The ethyl acetate extracts may be subjected to paper chromatography to add greater specificity to the method. With butanol saturated with 0.1 *N* NH_4OH as the solvent, hydrochlorothiazide has an R_f of 0.62 by the descending method. The spots may be visualized with short wavelength ultraviolet light (265 mμ) and eluted from the paper with acetone. After evaporation of the acetone, the chromotropic acid reaction may be carried out.

DISCUSSION

Hydrochlorothiazide and chlorothiazide are hydrolyzed by acid or alkali to yield 4-amino-6-chlorobenzene-1,3-disulfonamide. In the Bratton-Marshall reaction the 4-amino group is diazotized with nitrous acid and then coupled with N(1-naphthyl) ethylene diamine. One way to distinguish the two drugs is by varying the time of hydrolysis. Chlorothiazide gives maximum color after about five minutes, whereas the dihydro derivative requires at least thirty minutes. The possible use of sulfonamides along with these diuretics, however, would result in unusually high values.

The chromotropic acid reaction readily differentiates between the two benzothiadiazine compounds. In addition to the disulfonamide, chlorothiazide and hydrochlorothiazide yield formic acid and formaldehyde, respectively, on hydrolysis with acid. The formaldehyde but not the formic acid will then react with the chromotropic acid to give the characteristic purple color. This high degree of specificity plus sensitivity makes the reaction with chromotropic acid the preferred method for the analysis of hydrochlorothiazide.

The sensitivity of both methods is of the order of 10 mcg./ml. of urine when carried out as described.

SUMMARY

The application of the Bratton-Marshall reaction to the determination of hydrochlorothiazide has been described.

Another method, incorporating a high degree of specificity, involves the reaction of chromotropic acid with formaldehyde liberated during the acid hydrolysis of hydrochlorothiazide.

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Effect of a Lowered Catalase Level on Actions of Cardiac Glycosides*

By DUANE G. WENZEL and IVENS A. SIEGEL

Rats were given a high zinc diet to lower body catalase and simultaneously administered 0.5 mg./Kg. digitoxin daily for five weeks. The high zinc diet did not markedly alter the effect of digitoxin on serum or heart sodium or potassium. It did reduce the ECG abnormalities induced by digitoxin. The high zinc diet also reduced the mortality of an acute dose of ouabain for mice ($P < 0.05$) but again did not affect the heart sodium or potassium.

THE OBSERVATION that the unsaturated lactone ring of the cardiac glycosides is essential for the characteristic effects of these agents (1) has led to numerous studies of simple lactone moieties as possible cardiotonic drugs. While certain unsaturated lactones produce systolic standstill in the frog heart (2, 3), their effect on the mammalian heart has been both confirmed (4) and denied (5). The activity of the lactones appears to be related to the formation of a hydroperoxide (6). Hydrogen peroxide itself has been demonstrated to produce similar effects on the rat diaphragm (7) and digitoxin forms a peroxide that is highly effective in the inhibition of enzymes involved in cardiac metabolism (8).

A potentially important enzyme in the biological activity of peroxides is catalase. Among the better known actions of catalase are the decomposition of peroxides and the oxidation of alcohols and aldehydes. The report that the activity of the cardiac glycosides is unrelated to catalase (9) was based on a study conducted on frogs with compounds having questionable activity on the mammalian heart. It was therefore decided to re-examine the possible role of catalase in the functioning of the cardiac glycosides. The approach employed was to determine what, if any, changes in certain activities of the cardiac glycosides would be produced by an *in vivo* reduction of catalase (10).

EXPERIMENTAL

In order to determine the effect of catalase reduction on the chronic activity of digitoxin in the rat, it was first necessary to approximate a relatively nontoxic chronic dose of the drug. Digitoxin was administered in daily intraperitoneal doses of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg./Kg. to two rats per dose. All rats used throughout the study were black-hooded females weighing from 170 to 200 Gm. Digitoxin was administered for one week during which time daily ECG records were obtained under pentobarbitalization. Records were obtained with four standard leads by means of an Edin 8122A capacity coupled amplifier and a Brush BL-201 pen writing oscillograph. Because of the rapid heart rate, a paper speed of 125 mm./second was employed.

After seven days one animal in the 1.0 mg./Kg. dose group and all animals at larger doses had died. Marked weight loss and electrocardiographic alterations were prominent in these animals. The ECG changes consisted of an increased P-R interval, S-T segment depression, and an increase in QRS voltage. As all doses below 1.0 mg./Kg./day produced minimal weight and ECG effects, 0.5 mg./Kg./day of digitoxin was selected as the experimental dose.

Four groups consisting of ten rats per group were employed. One group served as control; the other three groups were placed, respectively, on digitoxin, zinc-fortified diet, and zinc plus digitoxin regimens. The zinc-fortified diet was prepared by mixing zinc carbonate with coarsely ground Purina laboratory chow to give a final zinc concentration of 0.5%. Electrocardiograms and serum sodium and potassium concentrations were obtained weekly for five weeks. Blood for the latter determination was withdrawn from the jugular vein while the animal was under anesthesia following electrocardiography. A Beckman model 41 direct reading flame photometer was used to determine the serum sodium and potassium. At the end of the fifth week all animals were sacrified, the hearts removed to determine the sodium and potassium content (11) and the livers removed to determine their catalase content (12).

The liver rather than the heart was analyzed for its catalase content as the liver, kidney, and erythrocytes are the principal locations of this enzyme. Evidence of its presence in other tissues has not only been questionable (13), but we were unable to determine any catalase activity in the rat myocardium. Furthermore, the well-known importance of the liver in the functioning of the mammalian heart (14) and the fact that injected digitoxin is quickly and persistently fixed in an active form in the rat liver (15) makes the liver the logical organ in which to determine catalase.

The preceding study was designed to provide information concerning the relationship of catalase to chronic, relatively nontoxic levels of digitoxin. The following procedure was planned to relate the action of catalase to the lethal dose of ouabain. The lethal range of ouabain was first approximated for male albino mice weighing between 30 and 40 Gm. This lethal range was then spanned with seven doses of digitoxin in a geometric progression of 1.5. Ten mice were used for each of the seven

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Abstracted from a portion of the data contained in a thesis submitted to the Graduate School of the University of Kansas by Ivens A. Siegel for the degree of Master of Science in Pharmacology.

This investigation was supported by a research grant from the University of Kansas.

doses. Seventy animals were fed the 0.5% zinc diet and an equal number the control diet for five weeks. The calculated doses of ouabain were then administered intraperitoneally to both groups. Sodium and potassium concentrations of all the experimental mouse hearts were determined at the termination of the experiment. The concentrations of these cations were also determined in a group of ten untreated controls.

RESULTS AND CONCLUSIONS

Electrocardiographic differences were observed between the digitoxin and zinc-digitoxin treated rats. The greatest and most consistent changes were observed in the amplitude of the QRS voltage and in the character of the S-T segment. While the control and zinc-treated groups displayed negligible alterations in the ECG, most of the digitoxin-treated rats displayed increased QRS voltage and a depressed S-T segment. A minimal QRS voltage increase and S-T depression were seen in a minority of the zinc-digitoxin animals.

The mean weekly serum sodium and potassium values are presented in Figs. 1 and 2. No significant change in sodium had occurred at five weeks although the sodium level of the digitoxin group had undergone a considerable fall. A marked fall in serum sodium was demonstrated in the zinc and zinc-digitoxin groups at the second week of treatment although both recovered much of the sodium loss during the last three weeks. The serum potassium values of the digitoxin groups were all significantly elevated ($P < 0.01$) above the control at the one-week period. At the two-week period the digitoxin ($P < 0.01$) and zinc-digitoxin ($P < 0.05$) were still elevated. At the termination of the experiment, however, all treated groups demonstrated a slightly-lowered but remarkably uniform serum potassium content. It is apparent from the graphs that the zinc diet, instead of modifying the effect of digitoxin on the serum sodium and potassium, appeared to influence the concentrations in the same general direction as the digitoxin.

Table I contains the mean heart sodium and potassium concentrations and the mean liver catalase activities. There were no significant changes in sodium and potassium concentrations. In both groups treated with zinc the catalase was reduced to about 60% of the concentration in the control or the digitoxin group. The digitoxin did not affect the catalase levels.

The mortality curves obtained with the ouabain- and the zinc-ouabain-treated mice are given in Fig. 3. When analyzed according to the method of Litchfield and Wilcoxon (16), the LD_{50}'s and 95% confidence limits are 7.5 mg./Kg. (5.59–10.05) for ouabain and 11.7 mg./Kg. (8.36–16.38) for zinc-ouabain. The difference of the LD_{50}'s was barely significant at the 0.05% level. The ranges of the mean heart sodium and potassium levels of all doses of the ouabain-treated mice were 4.36–5.04 and 7.19–7.98 meq., respectively. The ranges of the sodium and potassium levels for the zinc-ouabain hearts were 4.32–4.98 and 7.17–7.47, respectively. Mean values for the ten control animals were 4.62 for sodium and 7.47 meq. for potassium. It is apparent that neither the administration of ouabain nor the combined effect of ouabain and a zinc diet caused a significant alteration of the sodium and potassium concentrations. This is in contrast to a rather general agreement that toxic doses of the cardiac glycosides liberate potassium from the heart muscle (17).

The results as a whole are, at best, suggestive and

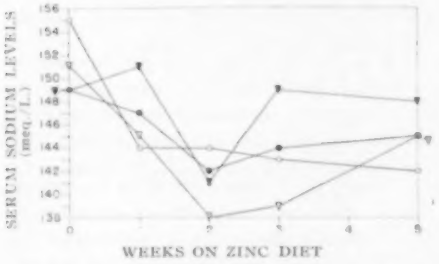

Fig. 1.—Mean weekly serum sodium values. ●, Control; ○, digitoxin; ▼, zinc; ▽, zinc and digitoxin.

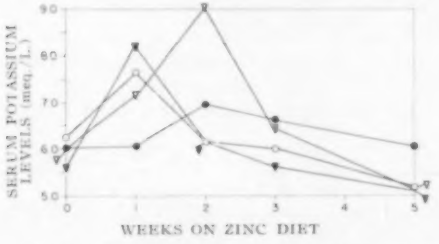

Fig. 2.—Mean weekly serum potassium values. ●, Control; ○, digitoxin; ▼, zinc; ▽, zinc and digitoxin.

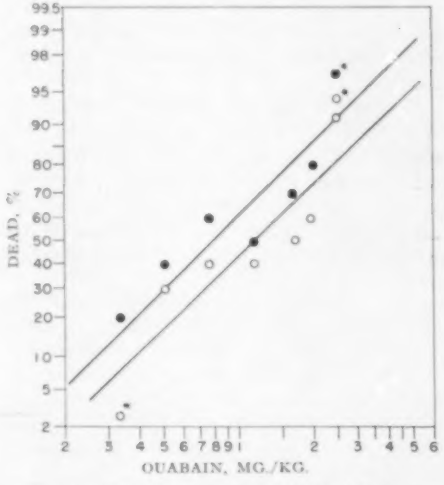

Fig. 3.—Mortality curves obtained with the ouabain and the zinc-ouabain treated mice. ●, Control; ○, zinc diet; ⊙, calculated points.

TABLE I.—MEAN SODIUM AND POTASSIUM CONTENT OF THE HEARTS AND CATALASE CONTENT OF THE LIVERS OF ZINC- AND DIGITOXIN-TREATED RATS[a]

Determination	Control	Digitoxin	Zinc Diet	Zinc-Digitoxin
Heart sodium[b]	5.226 ± 0.359	5.082 ± 0.168	4.464 ± 0.193	4.788 ± 0.678
Heart potassium[b]	7.124 ± 0.389	6.510 ± 0.125	6.410 ± 0.324	6.475 ± 0.225
Liver catalase[c]	0.789 ± 0.0502	0.793 ± 0.0235	0.504 ± 0.0179	0.505 ± 0.0640

[a] S. E. $\pm \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

[b] Meq./100 Gm. heart, wet weight.

[c] Meq. $NaBO_3 \cdot 4H_2O$/mg. liver, wet weight.

do not indicate any appreciable interaction between the cardiac glycosides tested and the catalase level. Even if marked changes in the action of the cardiac glycosides could be produced by a high zinc diet, one could not assume that the changes were a direct consequence of lowered catalase activity as they could be the result of some undefined effect of a zinc diet. With this limitation in mind, there were two results in which the zinc diet seemed to reduce the activity of the cardiac glycosides. It was quite apparent in the tendency to normalize the electrocardiograms of the digitoxin-treated rats. Likewise, the reduction in the mouse-ouabain mortality produced by the zinc diet, although barely significant, was in the same direction at each of the seven doses tested. It is quite possible that the mouse mortality may have been the result of toxicity to the brain as a number of the animals died in convulsive seizures. Rats have been reported to be especially susceptible to the CNS effects of rapidly administered cardiac glycosides (18). It is possible that the change in the mortality may be related in some manner to the facts that the cardiac glycosides stimulate oxygen uptake by the brain (19), the brain has a low catalase content and is susceptible to hydrogen peroxide (20).

SUMMARY

1. The catalase level of female black-hooded rats and male albino mice was lowered by the administration of a diet containing 0.5 per cent zinc.

2. Zinc-fed rats and controls were administered 0.5 mg./Kg./day of digitoxin for five weeks. Electrocardiograms and serum sodium and potassium were determined at weekly intervals. The hearts were analyzed for sodium and potassium and the liver for catalase at the end of the five-week period. The zinc diet reduced the rat liver catalase by 40 per cent and decreased the ECG abnormalities induced by digitoxin, but did not significantly alter the effect of digitoxin on serum or heart sodium or potassium.

3. The LD_{50} of ouabain was determined in mice treated with zinc for five weeks and in controls. The hearts were analyzed for sodium and potassium at the time of death. Treatment with zinc reduced the mortality of the mice treated with ouabain ($P < 0.05$) but did not affect the sodium or potassium content of the heart.

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Menispermaceae Alkaloids I*

The Alkaloids of *Cissampelos pareira* Linn. and the Origin of *Radix Pareirae Bravae*

By S. MORRIS KUPCHAN, NAOKATA YOKOYAMA, and JACK L. BEAL†

The roots and vines of *Cissampelos pareira* Linn. from Madras yielded *l*-curine, *d*-isochondrodendrine, and hayatin. Preliminary pharmacological study of the methanol-extractable alkaloids, of the methiodide prepared from the latter mixture, and of the quaternary alkaloids, showed that all had curare-like activity. The relationship of the composition and pharmacological activity of the alkaloidal mixture to the problem of the botanical source of the drug *radix pareirae bravae* is discussed.

CISSAMPELOS PAREIRA Linn. is a climbing shrub distributed throughout tropical and subtropical India and warm parts of Asia, East Africa, and America. The roots are reported to have found use as a diuretic, febrifuge, heart trouble remedy, and against dysentery and sores (1).

Wiggers in 1840 (2) isolated an amorphous alkaloid from the roots of a South American *C. pareira* sample, and the name pelosine was assigned to the alkaloid. Scholtz (3, 4) showed that pelosine is identical with *l*-curine (*l*-bebeerine[1]). Bhattacharji, Sharma, and Dhar reported in 1956 (5) that *C. pareira* Linn. from Kashmir yielded two new alkaloids, hayatin and hayatinin, and that the same species from Pilibhit yielded hayatin and *l*-curine, but no hayatinin. The methiodide of hayatin was shown to possess powerful neuromuscular blocking activity comparable to that of *d*-tubocurarine chloride (6).

The present report describes a preliminary study of the alkaloids of the roots and vines of *C. pareira* Linn. from Madras. Coarsely ground plant was extracted successively with petroleum ether, methanol, 1.5 per cent triethylamine in methanol, and 1.5 per cent hydrochloric acid solution. Each extract was processed for alkaloid content by the procedure summarized in Fig. 1. The crude fractions thus obtained were either crystallized directly, or subjected to chromatography on alumina, whereby separation into crystallizable fractions was effected. The isolation procedure afforded *l*-curine as the principal isolable alkaloidal constituent (1.2 per cent), and *d*-isochondrodendrine (0.2 per cent) and hayatin (0.005 per cent) as lesser isolable constituents. It is noteworthy that the plant from Kashmir yielded more hayatin (0.15 per cent) but no *l*-curine, whereas the sample from Pilibhit yielded *l*-curine (0.33 per cent) but neither hayatinin nor *d*-isochondrodendrine (5). Preliminary pharmacological study of our methanol-extractable alkaloids (fractions A, B, and C), of the methiodide prepared from the latter mixture, and of the quaternary alkaloids (fraction G) showed that all had curare-like activity.[2]

C. pareira was regarded for a great many years as the botanical origin of the drug *radix pareirae bravae*. In 1648, Piso (7) and Marcgrav (8) both described and figured a plant with a wide distribution in the New World known as *Caapeba*, which had a reputation in the treatment of calculus and other bladder complaints. The fact that *Caapeba* was used in the treatment of the same ailments for which *pareira brava* was used led to the early presumption that *Caapeba* was the botanical origin of *pareira brava*. Furthermore, Piso's description of the fruit of the *Caapeba* plant closely fitted the fruit of *Cissampelos pareira*. Linnaeus in 1763 gave the name *C. pareira* to *Caapeba* (9) and thereby lent the weight of his authority to the belief that *pareira brava* originated from *C. pareira*. Woodville (10) in 1790 listed *pareira brava* as a synonym for *C. pareira* and spoke of them interchangeably. Loudon (11) in 1841 also stated that *C. pareira* was the botanical origin of *pareira brava*. In four revisions of the United States Pharmacopeia beginning in 1842 (12–15), *C. pareira* Linn. was recognized as the origin of the drug pareira, the official name of *pareira brava*.

Hanbury (16) in 1873 reopened the question of the botanical origin of *pareira brava*. He compared samples of the drug from Brazil with authentic specimens of *C. pareira* Linn. from Jamaica, Trinidad, Ceylon, and Brazil and reported that in a histological study the samples of

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[1] The name curine is preferable to the ill-chosen term bebeerine. See the discussion by Wintersteiner, O., "Curare and Curare-Like Agents," Elsevier Publishing Co., Amsterdam, 1959, pp. 153–154.

[2] The authors wish to thank Mr. Edward Macko of the Smith Kline and French Laboratories, Philadelphia, Pa., for the pharmacological results reported herein.

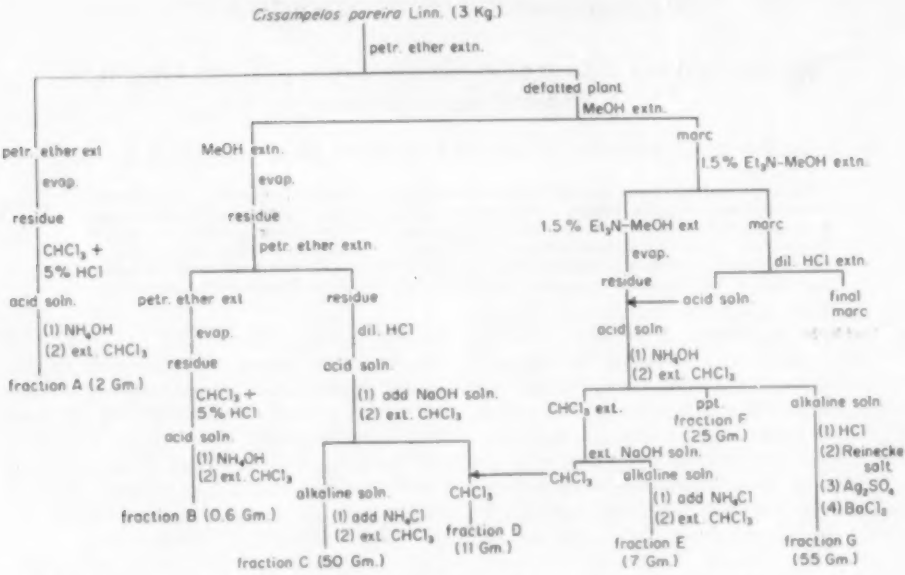

Fig. 1.—Flow sheet for separation of alkaloids of *Cissampelos pareira* Linn.

pareira brava differed notably from the *Cissampelos* specimens. Hanbury drew the conclusion that the pharmacologically-active *pareira brava* came from *Chondrodendron tomentosum*. He stated, however, that in England a drug called *pareira brava*, completely devoid of medicinal value, was being sold and he claimed that the English drug was *C. pareira*. Hanbury's work appears to have exerted a profound influence on the subject. The sixth revision of the United States Pharmacopeia (17) in 1882 listed *Ch. tomentosum* as the official botanical origin of *pareira*. In addition, many other authoritative publications accepted Hanbury's contention (18–25). More recently, Krukhoff and Moldenke (26) critically surveyed the American *Menispermaceae* and concluded that *Ch. platyphyllum* (St. Hil.) Miers is the major source of *pareira*.

In 1899, Scholtz (4) isolated *l*-curine from *pareira brava*. Somewhat later, Scholtz and Koch (27) examined a large quantity of *pareira brava* from a different commercial source and found only traces of *l*-curine. As a result of these recorded variations and of their own experiences, Faltis and Neumann (28) came to the conclusion that *Ch. platyphyllum* (St. Hil.) Miers is the true source of *pareira brava* but that unless roots from closely allied species exist which cannot be differentiated by the pharmacognosist, it may be that *Ch. platyphyllum* is subject to climatic and seasonal influences which determine the nature of the alkaloid content. The most significant chemical contribution to the field has been that of King in 1940 (29). King studied the alkaloids of *radix pareirae bravae* of the English market, of botanically identified *Ch. platyphyllum* (St. Hil.) Miers collected in Brazil in the region of Rio de Janiero and from Bahia, and of *Ch. microphyllum*, also collected near Bahia. The root of *Ch. platyphyllum* from Rio de Janiero contained *l*-curine and *d*-isochondrodendrine, and the same alkaloids were found in the root of the species collected near Bahia and identified as *pareira brava* by a local exporter of medicinal plants. *Ch. microphyllum* from Bahia contained *d*-curine and *d*-isochondrodendrine. *Radix pareirae bravae* of the English market also contained *d*-curine and *d*-isochondrodendrine in addition to a small amount of *l* isococlaurine. King concluded when *pareira brava* yields *l*-curine it comes from *Ch. platyphyllum* and when it yields *d*-curine, from *Ch. microphyllum*.

It is noteworthy that our study of *C. pareira* Linn. has demonstrated that the principal isolable alkaloids, *l*-curine and *d*-isochondrodendrine, are the same as the principal isolable alkaloids of *Ch. platyphyllum* (St. Hil.) Miers. The determination of the botanical source of *pareira brava* is beyond the scope of the present work, and indeed, the history of *pareira brava* is so complex that it may now be impossible to determine the original historical botanical source of the drug.

In the light of our findings concerning the isolable alkaloids and curare-like activity of *C. pareira* Linn., the conclusions of Hanbury (16), King (29), and others concerning the lack of medicinal value of the latter plant appear unwarranted. It appears reasonable that medicinally-useful *pareira brava* may have originated at various times in history from either *Cissampelos pareira* Linn. or the *Chondrodendron* species now regarded as the true sources.

EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Baird double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Paper chromatography was conducted by the descending technique on Whatman No. 1 paper.

Extraction of Alkaloids from *Cissampelos pareira*, Separation into Main Fractions.—Coarsely ground *C. pareira*[a] (air-dried roots and vines, 3 Kg.) from Madras was extracted continuously for four days with petroleum ether (Skelly B, b. p. 60–80°) in a Soxhlet-type extractor. Evaporation under reduced pressure left a semisolid residue (138 Gm.). A portion of the residue (10.3 Gm.) was dissolved in chloroform (100 cc.). The chloroform solution was washed with water (10 cc.) and was then extracted with 5% hydrochloric acid (three 50-cc. portions). The combined acid extract was washed with ether (three 20-cc. portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform, and the chloroform extracts were combined and concentrated to about 100 cc. The extraction into acid and reconversion to free base was repeated as above. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 150 mg. of fraction A (see Fig. 1).

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methanol, with a fresh charge of solvent at the end of two days. When the extraction was stopped, after five days, the extract returning to the pot yielded upon evaporation, a residue which did not give a positive test with Mayer's reagent. The methanol extract was concentrated under reduced pressure to a brown semisolid residue. This residue was triturated with petroleum ether (500 cc.) to remove remaining oil. Workup of this petroleum ether extract as above yielded 610 mg. of alkaloid fraction B. The petroleum ether-insoluble residue was next triturated with 1.5% hydrochloric acid (2 L.). The acid extract was washed with ether (three 500-cc. portions), made alkaline with ammonium hydroxide, and extracted with chloroform (5 L.); a small quantity of insoluble precipitate which separated at the interface was rejected. To separate the phenolic alkaloids, the chloroform solution was extracted with 1% sodium hydroxide solution (2 L.), and the strongly alkaline extract was washed with chloroform. The alkaline extract was treated with sufficient solid ammonium chloride to bring the pH to about 8.5, whereupon a pale yellow precipitate separated. The solution was extracted exhaustively with chloroform; the chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 50 Gm. of brown phenolic alkaloid fraction C. The chloroform washings were combined with the chloroform solution of nonphenolic alkaloids and with a chloroform solution of the corresponding fraction derived by triethylamine-methanol extraction (see below). The combined solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 11 Gm. of brown, semisolid, nonphenolic alkaloid fraction D.

The marc remaining from the methanol extraction was next extracted continuously with 1.5% trimethylamine in methanol for six days. When the extraction was stopped, the extract returning to the pot yielded, upon evaporation, a residue which did not give a positive test with Mayer's reagent. The marc remaining from this extraction was percolated with 1.5% hydrochloric acid (5 L.). The triethylamine-methanol extract was evaporated to dryness under reduced pressure, and the residue was triturated with the hydrochloric acid extract of the marc. The acid solution was washed with chloroform (three 1-L. portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform and the insoluble precipitate which remained at the interface was collected (fraction F, 25 Gm.). The chloroform solution was treated as above to separate a nonphenolic portion (combined into fraction D) and the phenolic alkaloid fraction E (7 Gm.). The aqueous layer was combined with all the other aqueous layers obtained during the fractionation procedure, acidified with hydrochloric acid, and treated with a saturated Reinecke salt solution. The dried Reineckate salt (136 Gm.) of the quaternary alkaloids was treated essentially by the method of Tomita and Kikuchi (30) to liberate the quaternary alkaloids. The salt was dissolved in acetone. After filtration of insoluble solids, the acetone solution was treated with saturated aqueous silver sulfate solution to complete precipitation. The silver Reineckate was removed by filtration, and the filtrate was treated with barium chloride solution to complete precipitation. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 55 Gm. of quaternary chloride fraction G. Studies of this fraction will be reported at a later date.

Isolation of Alkaloids—*Fraction A*.—Crystallization of the crude alkaloid fraction (150 mg.) from methanol afforded needles (108 mg.), m. p. 217–219°; $[\alpha]_D^{25}$ −293° (c 1.00, ethanol); $[\alpha]_D^{25}$ −317° (c 1.15, $CHCl_3$); $\lambda_{max}^{alc.}$ 280 mμ (ε 9,300); 285 mμ (ε 9,200). The melting point was not depressed on admixture with an authentic sample of *l*-curine.[b]

[a] The authors thank Dr. C. B. Sulochana, University Botany Laboratory, Madras, India, for confirming the identity of the plant, and Rajaranga and Co., Madras, India, for gathering and forwarding the dried plant materials to us.

[b] The authors thank Dr. James D. Dutcher, Squibb Institute for Medical Research, New Brunswick, N. J., for an authentic sample of *l*-curine.

The paper chromatographic behavior[5] and infrared spectrum in chloroform solution were identical with those of the authentic *l*-curine sample.

Fraction B.—Crystallization of the crude alkaloid fraction (610 mg.) from methanol gave 420 mg. of *l*-curine.

Fraction C.—Crystallization of the crude alkaloid fraction (50 Gm.) from methanol afforded 41.5 Gm. of a crude microcrystalline mixture, m. p. 209–211°. A portion of the microcrystalline mixture (1.105 Gm.) was chromatographed on Woelm "neutral" alumina (20 Gm.). The alkaloid was applied to the column as a solution in benzene and the column was developed by successive elution with benzene-chloroform, chloroform, chloroform-methanol, and finally with methanol. The various fractions were recombined according to their paper chromatographic behavior. Crystallization from methanol of the alkaloids recovered from the benzene, benzene-chloroform, and chloroform eluates gave *l*-curine. The chloroform-1% methanol and chloroform-3% methanol eluates gave a mixture of *l*-curine and a second higher melting alkaloid. Fractional crystallization from methanol ultimately gave 812 mg. of *l*-curine and 128 mg. of the second alkaloid, m. p. 318–319° (decompn.), $[\alpha]_D^{25}$ +59° (c 1.15, pyr.); $\lambda_{max.}^{0.1\ N\ HCl}$ 273.5 mμ (ε 4,500), 282 mμ (ε 4,400). The melting point was not depressed on admixture with an authentic sample of *d*-isochondrodendrine.[6] The paper chromatographic behavior[5] and infrared spectrum (KBr pellet) were identical with those of the authentic *d*-isochondrodendrine sample. Methylation with diazomethane afforded the known dimethyl ether of *d*-isochondrodendrine, cycleanine, m. p. 273–274°, $[\alpha]_D^{23}$ −8.5° (c 4.65, $CHCl_3$) $\lambda_{max.}^{abs.}$ 276 mμ (ε 4,000), 284 mμ (ε 3,800). The melting point was not depressed on admixture of an authentic sample of cycleanine.[6] The paper chromatographic behavior and infrared spectrum in chloroform were identical with those of the authentic cycleanine sample.

Fraction D.—Chromatography of the alkaloid fraction by the procedure described above yielded 2.70 Gm. of *l*-curine, 110 mg. of *d*-isochondrodendrine, and 105 mg. of a third, high melting compound. Recrystallization of the third compound from aqueous pyridine gave colorless microcrystals, m. p. 301–302° (decompn.); $[\alpha]_D^{25}$ 0° (c 1.02 pyr.); $\lambda_{max.}^{0.1\ N\ HCl}$ 282 mμ (ε 8,200). The melting point was not depressed on admixture with an authentic sample of hayatin.[7] The paper chromatographic behavior[5] and infrared spectrum (KBr pellet) were identical with those of the authentic hayatin sample.

Fraction E.—Crystallization of the alkaloid fraction (7 Gm.) from methanol gave 2.8 Gm. of a crude microcrystalline mixture, m. p. 260–265°. Chromatography of a portion (1.10 Gm.) on neutral alumina (20 Gm.) by the procedure described above gave 387 mg. of *l*-curine, 440 mg. of *d*-isochondrodendrine, and 210 mg. of hayatin. The paper chromatographic behavior[5] of the noncrystalline fractions indicated the presence of *l*-curine as a principal component.

Fraction F.—Extraction of this highly insoluble fraction in a Soxhlet extractor with methanol for two days followed by concentration of the methanol extract yielded 2.46 Gm. of *l*-curine. Further extraction with pyridine failed to give any additional alkaloidal material.

PHARMACOLOGICAL RESULTS

Preliminary pharmacological tests were performed on the total methanol-extractable alkaloids (fractions A, B, and C), on the methiodide prepared therefrom, and on the quaternary alkaloid fraction.[2] The nonquaternary bases produced only bradypnea and moderate decreased activity after doses of 2,000 mg./Kg. orally in the mouse. Intraperitoneally, these alkaloids produced prolonged depression of motor activity after doses as low as 50 or 100 mg./Kg., and lethal effects at 250 mg./Kg. This material differed slightly from the methiodide or *d*-tubocurarine. With the latter two, no overt effects or transient CNS depressing effects are observed below lethal doses. With the nonquaternary bases, parenteral doses below the lethal dose caused depression for more than eight hours. After an acute intravenous dose of 2.5 to 10.0 mg./Kg. in the pentobarbitalized cat, the nonquaternary bases produced respiratory arrest. Hypotension was accompanied by respiratory depression at nontoxic dose levels. Only the responses to peripheral vagal stimulation were blocked or diminished. No other significant autonomic changes were observed.

The methiodide mixture produced exophthalmia, depression of motor activity, dyspnea, asphyxial convulsions, and acute death in mice after oral doses of 1,000 mg./Kg. Lower doses failed to produce overt biological activity. Intraperitoneally, this material caused absence of grasp reflex, asphyxial convulsions, and acute death after doses as low as 10 mg./Kg. No overt effects occurred at 1.0 mg./Kg. The methiodide caused hypotension in pentobarbitalized cats, but a very narrow range exists between the effective dose level and the toxic dose level.

The quaternary alkaloid fraction produced slight motor depression in mice after an oral dose of 500 mg./Kg. Larger doses caused marked depression and toxic manifestations with death at the 2,000 mg./Kg. dose level. Observed gross effects in mice: at 250 mg./Kg. p. o., no overt effects; at 500 mg./Kg. p. o., slight depression; at 1,000 mg./Kg. p. o., marked depression, low posture, dyspnea, slight atoxia, intention tremors, diarrhea (1/2); at 2,000 mg./Kg. p. o., dyspnea, marked depression, intention tremors, retching, exophthalmia, cyanosis, asphyxial convulsions, death (eight to twelve minutes). No significant alterations in pain threshold, pupil size, or body temperature were noted at the tested dose levels. In cats anesthetized with chloralose, the quaternary alkaloid fraction produced significant and sustained lowering of mean arterial blood pressure after acute intravenous dosage of 2.5

[5] The procedure and solvent system used were essentially those of Tomita and Watanabe (31). The method involved the use of paper pretreated with buffer at pH 3.5 and the detection of alkaloidal spots with a chloroform solution of bromophenol blue. The solvent system was the upper layer of a mixture of *n*-butanol: acetic acid: water (67:10:23 by volume) prepared by shaking well and allowing to stand at room temperature for two days.

[6] The authors thank Professor Masao Tomita, Kyoto University, Japan, for authentic samples of *d*-isochondrodendrine and its O,O-dimethyl ether, cycleanine.

[7] The authors thank Dr. S. Bhattacharji, Central Drug Research Institute, Lucknow, India, for an authentic sample of hayatin.

mg./Kg. (cumulative 4.0 mg./Kg.). Larger doses of 5 or 10 mg./Kg. proved lethal due to respiratory arrest. No autonomic effects were noted. None of the alkaloid fractions showed significant diuretic activity in the rat.

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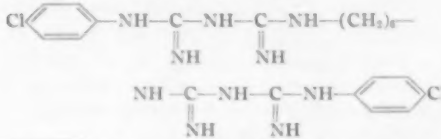
Antimicrobial Activity, *In Vitro*, of Chlorhexidine*

By C. A. LAWRENCE

Comparative *in vitro* tests indicate that chlorhexidine has greater antibacterial activity than benzalkonium chloride, a combination of *o*-phenyl- and *p*-*tert*-amylphenol, and an iodine-polyvinylpyrrolidone complex under the test conditions. Chlorhexidine in relatively high dilutions was effective against several cultures of Gram-negative bacteria in the phenol coefficient test. In moderate concentrations, it was active against staphylococci and anaerobes.

AN INCREASING number of reports in the British and Canadian literature describe the strong antimicrobial activity and clinical properties of a new germicide chlorhexidine, bis(*p*-chlorophenyl-diguanido)hexane,[1] which has the following chemical configuration



The compound is a colorless, odorless, strongly basic, highly insoluble salt with a melting point of 134°. The diacetate substance has a melting point of 154° and is soluble to approximately 1.9 per cent in water, whereas the dihydrochloride has a melting point of 275° and has a solubility of only 0.06 per cent (1).

As with other germicides, chlorhexidine is reduced somewhat in antimicrobial activity by certain organic substances, i. e., milk, blood, pus, etc.; however, this reduction is less marked than that observed with some of the other commonly used germicides (2). In mouse injection experiments, the compound still exerts its full effect when suspended in 0.2 per cent soap, is compatible with nonionic and cationic detergents (1),

* Received December 18, 1959, from the Los Angeles County Health Department and the Department of Infectious Diseases, University of California Medical Center, Los Angeles.

[1] Hibitane, Imperial Chemical (Pharmaceuticals) Ltd., England; available in the United States from Ayerst Laboratories, Inc., New York, N. Y.

TABLE I.—LIMITING DILUTIONS OF ANTIBACTERIAL AGENTS SHOWING BACTERIOSTATIC (BS) AND BACTERICIDAL (BC) ACTIVITIES AGAINST VARIOUS BACTERIA IN NUTRIENT MEDIA

Organisms	Disinfectant: Limiting Dilution Bacteriostatic (BS) and Bactericidal (BC) — Agent I[a] BS	Agent I[a] BC	Agent II[b] BS	Agent II[b] BC	Agent III[c] BS	Agent III[c] BC	Agent IV[d] BS	Agent IV[d] BC
Salmonella typhosa	600,000	600,000	80,000	80,000	1,000	1,000	4,000[e]	2,000
Pseudomonas aeruginosa	100,000	100,000	10,000	10,000	100	80	4,000[e]	4,000
Proteus vulgaris	80,000	80,000	40,000	40,000	2,000	2,000	4,000	4,000
Escherichia coli	800,000	800,000	60,000	60,000	1,000	1,000	2,000	2,000
Staphylococcus aureus No. 209	1,000,000	1,000,000	800,000	600,000	6,000	6,000	6,000	6,000
Staphylococcus aureus No. 261[f]	1,000,000	1,000,000	1,000,000	1,000,000	4,000	4,000	10,000	10,000
Bacillus cereus	400,000	80,000	200,000	200,000	10,000	10,000	6,000	4,000
Bacillus subtilis	1,000,000	1,000,000	800,000	800,000	8,000	8,000	4,000	4,000
Clostridium novyi	800,000	100,000	100,000	60,000	20,000	4,000	10,000	<1,000[e]
Clostridium tetani	2[illegible],000	200,000	200,000	200,000	10,000	10,000	6,000	6,000
Clostridium botulinum	400,000	200,000	200,000	200,000	10,000	10,000	6,000	6,000
Clostridium perfringens	100,000	100,000	200,000	200,000	6,000	6,000	2,000	2,000

[a] Chlorhexidine. [b] Benzalkonium chloride. [c] A phenolic type disinfectant. [d] An iodine-polyvinylpyrrolidone complex. [e] Dilutions based upon iodine content of iodine-PVP complex. [f] An antibiotic resistant strain of phage type 52, 42B, 44A, 81. [g] Highest concentration tested failed to show bactericidal activity.

TABLE II.—LIMITING DILUTIONS OF ANTIBACTERIAL AGENTS SHOWING FUNGISTATIC (FS) AND FUNGICIDAL (FC) ACTIVITIES AGAINST FUNGI IN NUTRIENT MEDIUM

Organisms	Disinfectant: Limiting Dilution Fungistatic (FS) and Fungicidal (FC) — Agent I[a] FS	Agent I[a] FC	Agent II[b] FS	Agent II[b] FC	Agent III[c] FS	Agent III[c] FC	Agent IV[d] FS	Agent IV[d] FC
Microsporum audouini	100,000	100,000	100,000	100,000	60,000	40,000	10,000[e]	10,000
Microsporum gypseum	100,000	100,000	80,000	80,000	10,000	10,000	4,000	4,000
Microsporum canis	80,000	80,000	100,000	100,000	40,000	40,000	8,000	8,000
Trichophyton tonsurans	100,000	100,000	100,000	100,000	10,000	10,000	8,000	8,000
Trichophyton mentagrophytes	600,000	600,000	400,000	400,000	60,000	40,000	40,000	40,000

[a], [b], [c], and [d] See footnotes Table I. [e] Dilutions based upon iodine content of iodine-PVP complex.

and has been used successfully in creams with penicillin (3, 4), neomycin, and bacitracin (5). In effective germicidal concentrations the compound has been reported to have little or no untoward effects in acute and chronic toxicity studies in animals (1), and is well tolerated when applied topically to the skin of humans (2, 5–8).

Chlorhexidine has been used successfully in reducing the incidence of postoperative infections by the treatment of instruments (2, 9, 10), disinfection of urine bottles (11), bedpans (12), and rubber gloves (13). It has also been suggested for use as a spray to prevent the spread of dust-borne infections in hospitals (14).

The compound, incorporated in a cream base, is being used extensively in the British Commonwealth to control staphylococcal infections in hospitals. A hand cream containing the germicide was found effective when used by physicians and nurses to reduce the incidence of infections in maternity wards and nurseries (15-20). A cream has also been applied effectively to the skin of infants in the prevention of staphylococcal infections (21). In combination with certain antibiotics, chlorhexidine in a cream base, when applied to the nasal cavities of staphylococcal carriers, has been considered an effective means for controlling the incidence of infections in hospital wards and also in persons outside of hospitals who were found to have recurrences of minor staphylococcal infections from nasal colonies of *Staphylococcus aureus* (5, 22).

Aqueous and alcoholic solutions of chlorhexidine have also been used successfully in burns (6), in dermatology for the treatment of fungal diseases (21, 23), in obstetrics and gynecology

(2, 21), as a skin antiseptic for preoperative preparation of the patient's skin, and for the surgeon's hand rinse (7, 8, 24, 25).

At the present writing, there have been but few references to the suggested use of chlorhexidine as a germicide published in the American literature (26). It is for this reason that a study was undertaken to determine the comparative antimicrobial properties, *in vitro*, of chlorhexidine with those of three other classes of disinfectants in common use in this country. The latter agents included a quaternary ammonium germicide, benzalkonium chloride,[2] a phenolic type disinfectant,[3] and an iodine-polyvinylpyrrolidone complex.[4]

EXPERIMENTAL

Bacteriostatic and Bactericidal Tests.—Primary dilutions of the disinfectants were made in distilled water and 1 ml. of the appropriate dilution added to 9 ml. of sterile beef extract broth in the studies in which the aerobic bacteria were tested. Brewer's fluid thioglycollate medium was used for the anaerobes (Clostridia). Each tube was inoculated with a loopful of a twenty-four-hour broth culture of test organism. The tubes were incubated at 37° for seventy-two hours and the presence or absence of visible growth recorded. Tubes showing no growth at this time were checked for bactericidal activity by transferring three loopfuls of the test mixture to tubes of sterile medium without added disinfectant. The latter tubes were incubated for an additional seventy-two hours. Failure of growth to occur in the subculture tubes was taken as evidence that the organisms had not survived or had been killed in the original broth-disinfectant tubes. The results of this study are presented in Table I.

Results.—From the data given in Table I it will be noted that of the four disinfectants tested, chlorhexidine in general gives an overall antibacterial activity greater than the other three germicides tested. In some instances the bacteria were affected by comparable concentrations of benzalkonium chloride and chlorhexidine. This was particularly true with the antibiotic resistant strain of *Staphylococcus aureus* (No. 261) and some of the anaerobes.

Under the same testing conditions, the phenolic disinfectant was found to be far less active than chlorhexidine and benzalkonium chloride against all of the organisms examined in the presence of the nutrient (proteinaceous) media. In general, the iodine-containing germicide showed an activity comparable with that of the phenolic preparation.

Fungistatic and Fungicidal Tests.—One milliliter of the distilled water dilutions of the disinfectants was added to 9 ml. of Neopeptone medium consisting of glucose, 4 Gm.; Bacto Neopeptone, 1 Gm.; distilled water 100 ml.; pH, 6.0. The fungi were first grown for a period of one month at room temperature in four-ounce screw-capped vials containing glass beads and the medium described above. At the end of the incubation period, the heavy mycelial and spore mass was shaken vigorously with the glass beads and the suspension filtered through several layers of sterile gauze. A loopful of this suspension was added to each tube of broth-disinfectant solution.

The tubes were incubated for one month at room temperature and those showing no growth at this time were checked for fungistatic activity by subculturing three loopfuls to tubes of medium without disinfectant. The latter were again incubated for one month and the presence or absence of visible growth recorded. The results of this study are presented in Table II.

Results.—Examination of the data given in Table II will reveal a close correlation in fungistatic and fungicidal activities between chlorhexidine and benzalkonium chloride, similar to the comparable effects of these two germicides against bacteria (Table I). The phenolic preparation is somewhat lower in antifungal properties than the latter two agents but shows a greater action against these organisms than against bacteria. In descending order of activity, an iodine-polyvinylpyrrolidone complex will be noted to have the least activity against the fungi studied.

Phenol Coefficient Test.—The A.O.A.C. (27) method for determining phenol coefficients was used in determining the germicidal activity of chlorhexidine. The tubes containing the dilutions of the latter agent and tubes of phenol were maintained in a water bath at 20° during the time intervals of transfer. In all instances the inoculum consisted of twenty-four-hour cultures of the respective organisms grown at 37°. The results of this study are presented in Table III.

Results.—In summarizing the data given in Table III it will be noted that chlorhexidine, in general,

TABLE III.—PHENOL COEFFICIENT (A. O. A. C. METHOD) OF CHLORHEXIDINE AGAINST A VARIETY OF BACTERIA[a]

Organisms	Limiting Dilutions Effective in Ten Minutes at 20°		Phenol Coefficient
	Chlorhexidine	Phenol	
Salmonella typhosa	1:40,000	1:120	333
Pseudomonas aeruginosa	1:15,000	1:100	150
Proteus vulgaris	1:5,000	1:100	50
Escherichia coli	1:25,000	1:100	250
Staphylococcus aureus No. 209	1:10,000	1:80	125
Staphylococcus aureus No. 261[b]	1:7,500	1:70	107
Bacillus cereus	1:70,000	1:130	538
Bacillus subtilis	1:5,000	1:50	100
Clostridium novyi	1:5,000	1:20	250
Clostridium tetani	1:5,000	1:140	36
Clostridium botulinum	1:2,500	1:190	13
Clostridium perfringens	1:2,500	1:130	19

[a] All of the cultures used in this test were incubated at 37° for twenty-four hours prior to testing. The clostridia were cultured and transferred in fluid thioglycollate medium. All of the remaining organisms were grown in beef extract broth.

[b] See footnote *f*, Table I.

[2] Zephiran chloride, Winthrop-Stearns Inc., New York, N. Y.

[3] Amphyl (o-phenylphenol and *p-tert*-amylphenol), Lehn and Fink Products Corp., New York, N. Y.

[4] Isodine, Isodine Pharmacal Corp., New York, N. Y.

shows a high germicidal activity against the Gram-negative bacteria, with dilutions ranging from 1:15,000 (*Pseudomonas aeruginosa*) to 1:40,000 (*Salmonella typhosa*) effective in ten minutes. The one exception in this group of bacteria was *Proteus vulgaris* which required a 1:5,000 dilution to effect complete destruction of this organism. The effective germicidal range of chlorhexidine against the two strains of *S. aureus* was between 1:7,500 to 1:10,000.

While the strain of *Bacillus cereus* used in this study appeared to be relatively resistant to chlorhexidine in the broth dilution test (Table I), the culture was highly sensitive to the disinfectant when tested in an aqueous dilution of the germicide. As noted in Table III, a dilution of 1:70,000 was effective against this organism. Also, a dilution of 1:130 phenol proved to be germicidal for this organism. *Bacillus subtilis*, on the other hand was considerably more resistant to both germicides, since concentrations of 1:5,000 and 1:50 of chlorhexidine and phenol, respectively, were required to destroy this organism.

The twenty-four-hour cultures of the anaerobes required concentrations of 1:2,500 to 1:5,000 of chlorhexidine for complete destruction. With the exception of the strain of *Clostridium novyi*, which required a concentration of 1:50 phenol for effective kill, the remaining clostridia failed to grow after being exposed to dilutions ranging from 1:130 to 1:190 of the latter germicide.

SUMMARY AND CONCLUSIONS

The present report compares the antimicrobial activity, *in vitro*, of chlorhexidine with that of a quaternary ammonium disinfectant, a phenolic compound, and an iodophor. In general, the new compound showed greater antibacterial activity than the other classes of disinfectants when tested in the presence of nutrient media. It was also found to be highly effective against several strains of dermatophytes.

Using the phenol coefficient test, chlorhexidine, in relatively high dilutions, was found to be effective against several cultures of Gram-negative bacteria. In moderate concentrations, the compound was found to be active against staphylococci and anaerobes.

Reference is made to a number of British publications in which chlorhexidine has been reported to be an effective adjunct in reducing the incidence of postoperative infections and the control of staphylococcal infections in hospitals.

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Notes

Note on Saponins and Their Sapogenins from Strawberry Clover*

BY E. D. WALTER

A crystalline mixture of saponins was isolated from strawberry clover (*Trifolium fragiferum*). Upon hydrolysis with dilute acid this product yielded the soyasapogenols B and C, a very small quantity of unidentified sapogenin, and several sugars.

SOYASAPOGENOLS B and C and possibly A have previously been isolated from the saponins of alfalfa and ladino clover (1) suggesting a rather frequent occurrence of these sapogenols in legume forages.

EXPERIMENTAL

Fresh strawberry clover (45 Kg.) was placed in a stainless steel tank and covered with 95% alcohol (50 gallons) and allowed to stand for five days. A 2-gallon portion was drained, filtered, and concentrated to about one-fourth volume when solids appeared. The material was transferred to a separatory funnel and shaken gently with about one-third volume of ether. The saponin separated in the aqueous layer as minute crystals. This layer was centrifuged and the residue was washed with water, and finally with acetone. The yield of dry saponin was about 1 Gm. or about 0.3% of the dry weight of clover.

With another 2-gallon portion of the extract the saponin was isolated the same way except that the extract was treated with about 40 Gm. of charcoal per gallon and filtered through filter aid. This method required less washing with acetone to remove green material, but the yield was about the same. Both methods were employed to obtain the saponin stock for subsequent studies.

The dry saponin was practically insoluble in water, but appreciably soluble in 50–60% ethanol. When concentrated sulfuric acid was added to the dry material a red color formed almost instantly. This rapid color reaction was found particularly characteristic of the unidentified sapogenin melting at 321°.

No action resulted from contact of this saponin with an isolated strip of rabbit ileum, possibly because of its insolubility in the Tyrode's solution in which the strip was suspended. This is in contrast with the results from alfalfa and ladino saponin (1) and from bur clover saponin (hederin) (2).

Isolation of Sapogenins.—The saponin (0.77 Gm.) was dissolved in 60% ethanol and sulfuric acid was added to make the solution approximately 1 *N*. The total volume was about 108 ml. This solution was refluxed for sixty hours. Water was added to precipitate the sapogenins and they were then extracted with ether in a separatory funnel. The ether solution was washed with water to remove sulfuric acid, dehydrated with anhydrous sodium sulfate, and clarified with charcoal. The ether was evaporated and the sapogenins were crystallized from methanol; yield 0.344 Gm. About 45% of the saponin was sapogenin. This method was used to obtain larger quantities of sapogenin.

The acid was removed from the hydrolysate as barium sulfate, and the filtrate from this was used for chromatographic identification of the sugars. The following sugars were detected: glucose, galactose, arabinose, xylose, rhamnose, and glucuronic acid, with xylose predominating. The large number of sugars present suggested that more than one saponin was involved.

The sapogenins were first separated into chloroform-soluble and insoluble fractions. Roughly 90% of the material was soluble in chloroform, while the remainder was largely the high melting (321°) fraction, which gave the rapid color reaction with sulfuric acid.

The chloroform-soluble portion, after crystallization from methanol, was dissolved in benzene and chromatographed on a column of alumina (deactivated with 10% acetic acid in benzene), and eluted with increasing concentrations of methanol in benzene (0.5, 2.5, and 100% methanol). The eluate from 0.5% methanol contained material melting at 235–240° and corresponding to soyasapogenol C. The 2.5% methanol eluate contained a fraction melting at 255–260°, corresponding to soyasapogenol B. The 100% methanol fraction contained a trace of the material melting at 321°.

Soyasapogenol B.—The fraction melting at 255–260° was recrystallized from methanol-chloroform yielding material of m. p. 260°, $[\alpha]_D^{25}$ + 90.7°.

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.55; H, 10.99. Found: C, 78.80; H, 10.6.

Acetylation of this material and crystallization from methanol-chloroform furnished the triacetate m. p. 180°, $[\alpha]_D^{25}$ + 78°.

Anal.—Calcd. for $C_{36}H_{54}O_6$: C, 73.93; H, 9.65. Found: C, 74.0; H, 9.49.

The infrared spectra of this triacetate and that of authentic soyasapogenol B triacetate were identical.

Soyasapogenol C.—Recrystallization of the fraction melting at 235–240° in methanol-chloroform yielded a product of m. p. 240°, $[\alpha]_D^{25}$ + 66°. Meyer, *et al.* (3), reported m. p. 239–240° and $[\alpha]_D^{25}$ + 65°.

Acetylation of this sapogenol with acetic anhydride and chromatographing on deactivated alumina, followed by elution with petroleum ether-benzene (3:1), and crystallization from methanol furnished

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The author is indebted to Arthur Bevenue for paper chromatography of the sugars, to Lawrence White and Geraldine Secor for elemental analyses, and to Edith Gong for the infrared spectra.

the diacetate m. p. 204°, $[\alpha]_D^{25}$ + 59°. The infrared spectra of this material and that of authentic soyasapogenol C diacetate were identical.

Anal.—Calcd. for $C_{34}H_{52}O_4$: C, 77.82; H, 9.99. Found: C, 77.8; H, 9.79.

Unidentified Sapogenin.—The minor fraction eluted from the alumina column with methanol melted at 321°, and had a $[\alpha]_D^{25}$ + 97°. This material was acetylated in pyridine solution with acetic anhydride. The product was put on a deactivated alumina column with Skellysolve-benzene (3:1), and was eluted with Skellysolve-benzene (1:1), followed by benzene. The benzene eluate, when crystallized from methanol and dried in a vacuum at 121°, melted at 227–228°; $[\alpha]_D^{25}$ − 35.5°. Infrared spectra of the sapogenin $\lambda_{max.}^{KBr}$ 5.87 μ (and strong hydroxyl bands), and of the acetate $\lambda_{max.}^{KBr}$ 5.76, 5.86, and 8.1 μ indicated the presence of a carbonyl group. The ultraviolet absorption spectra for the acetate $\lambda_{max.}^{EtOH}$ 241.5 mμ, ϵ 23,000, 249 mμ, ϵ 25,900, 258 mμ, ϵ 20,800 indicated a conjugated system. Both compounds gave a yellow color with tetranitromethane, and, in contact with concentrated sulfuric acid, gave a red color instantly.

Anal.—Calcd. for $C_{36}H_{54}O_7$: C, 72.2; H, 9.07; for three CH_3CO groups, 21.5; mol. wt., 598.72. Found: C, 72.4; H, 8.72; CH_3CO, 21.1; mol. wt. (Rast), 612.

Insufficient quantity of material prevented further characterization of this sapogenin at this time.

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Note on the Hydrophile-Lipophile Balance of Tragacanth*

By WALLACE L. GUESS

IN A RECENT publication, Chun, *et al.* (1, 2), reported on their investigation of the HLB of some naturally occurring emulsifying agents. These workers pointed out that while tragacanth should be studied more extensively, they did obtain an HLB value for this product of 13–13.5. This value was obtained by using Span[1] 20 and Span 80 as reference surfactants. It was noted in our laboratories that satisfactory oil-in-water emulsions could not be produced using the Spans and tragacanth. Therefore, the following work was undertaken.

EXPERIMENTAL

The method of Chun, *et al.*, for the determination of the HLB of tragacanth was duplicated as nearly as possible with a modification of the method of shaking the emulsions. In this work, all emulsions were shaken for five-minute intervals on a Red Devil paint conditioner at 1,600 oscillations per minute. Every emulsion was shaken for three such periods: (*a*) once immediately after mixing all components, (*b*) once at the end of one hour, and (*c*) finally at the end of twenty-four hours. The emulsions were allowed to stand undisturbed for twenty-four hours and were evaluated by a blind technique (simple masking of labels) according to degree of creaming and/or oil separation. In order to insure that only oil-in-water emulsions were being evaluated, a drop-dilution test (3) and a water-soluble dye test (3) were conducted on each emulsion.

The results of this study are shown in Table I.

TABLE I.—HLB VALUES OF TRAGACANTH

Tragacanth	HLB of Tragacanth with Tween 20	Tween 40	Tween 60	Tween 80
Meyer Bros., lot 163607	11.62	11.90	11.92	11.84
Meer, lot No. 10	12.52	11.90	11.92	11.76
Average HLB	12.07	11.90	11.92	11.80

DISCUSSION

It is interesting to note that in these laboratories no o/w emulsions could be prepared with combinations of Spans and tragacanth, while all the Tweens used in optimum proportions with tragacanth produced smooth, white, slowly-creaming o/w emulsions. These observations were true with two different lots of tragacanth. It may also be observed that an average HLB value of 11.92 was determined, which checks fairly well with the value obtained by Chun and his co-workers, particularly since a natural product of such varied physical characteristics as tragacanth was used.

SUMMARY

It is recommended that further investigation into the HLB of tragacanth be undertaken to determine if various lots of tragacanth would show a range of HLB values even greater than those referred to in this paper. It may be of interest to determine if tragacanth has an HLB in the true sense as do the synthetic surfactants, or if an HLB value is only an apparent value dependent on viscosity of the tragacanth, per cent insoluble bassorin, and other well-known factors.

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Note on Triazinylmethylureas. Guanamines VII*

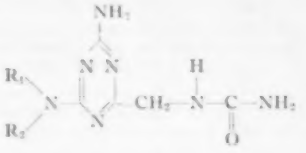
By SEYMOUR L. SHAPIRO, VINCENT A. PARRINO, and LOUIS FREEDMAN

Condensation of ethyl hydantoate with substituted biguanides gave triazinylmethylureas (I) which are pharmacologically active.

MANY urea derivatives have afforded useful pharmacological effects (1), and in this study a series of triazinylmethylureas (I) envisioned as structural analogs of phenacemide (II) (2) have been synthesized. Diphenylhydantoin, interestingly embodies the aralkylurea structural features of I, and the acylurea element of II.

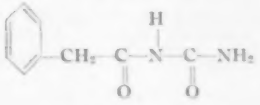
The compounds were prepared by condensation (3) of ethyl hydantoate with the appropriate biguanide (4, 5) which afforded the desired I in 5–38% yields of purified product (6).

On pharmacological evaluation (7), compounds 1 and 5 (anticonvulsant), 11 (analgesic), and 3 (anti-inflammatory) gave significant responses.



I

R_1 = alkyl, aralkyl, aryl
R_2 = H, lower alkyl



II

TABLE I.—TRIAZINYLMETHYLUREAS[a] (SEE FORMULA I)

No.	R_1	M. p., °C.[b]	Sc	Formula	Analyses, %[d] Carbon Calcd.	Carbon Found	Hydrogen Calcd	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	C_2H_5—[e]	202–203	A	$C_6H_{13}N_7O$	43.0	43.1	5.9	5.8	...	...
2	[f]	231	A	$C_{10}H_{17}N_7O$	47.8	48.1	6.8	7.1	...	...
3[g]	$C_6H_5CH_2$—	183–184	B	$C_{12}H_{19}N_7O_2$[h]	...	...	...	...	32.1	32.7
4	$C_6H_5CH_2CH_2$—	204–205	A	$C_{12}H_{17}N_7O$	...	...	...	...	34.1	33.8
5[i]	C_6H_5—	227	C	$C_{11}H_{15}N_7O$	51.0	51.4	5.1	5.0	37.8	37.6
6[g]	C_6H_5—	238	B	$C_{12}H_{15}N_7O$	52.7	53.2	5.5	5.9	35.9	36.3
7	2—$CH_3C_6H_4$—	209–211	B	$C_{12}H_{15}N_7O$	52.7	52.2	5.5	5.4	35.9	35.5
8[g]	2—$CH_3C_6H_4$—	247–248	B	$C_{12}H_{17}N_7O$	54.3	54.3	6.0	6.0	34.1	34.4
9[j]	4—$CH_3C_6H_4$—	213–214	B	$C_{14}H_{19}N_7O$	55.8	55.4	6.4	6.1	...	...
10	2,6—$(CH_3)_2C_6H_3$—	250	A	$C_{13}H_{17}N_7O$	...	...	...	...	34.1	34.2
11	2—ClC_6H_4—	169	D	$C_{11}H_{12}ClN_7O$	...	...	...	...	33.4	33.1
12	4—ClC_6H_4—	257	A	$C_{11}H_{12}ClN_7O$	45.0	44.9	4.1	3.7	33.4	33.5
13	2—BrC_6H_4—	295	A	$C_{11}H_{12}BrN_7O$	...	...	...	...	29.0	28.5
14	3—BrC_6H_4—	218	A	$C_{11}H_{12}BrN_7O$	39.1	39.5	3.6	3.8	29.0	28.6
15	4—$CH_3OC_6H_4$—	216–217	B	$C_{12}H_{15}N_7O_2$	49.8	50.1	5.2	5.0	...	...

[a] R_2 = H unless otherwise indicated. [b] Melting points are not corrected and the compounds melted with decomposition. [c] Recrystallizing solvent; A = dimethylformamide + acetonitrile; B = dimethylformamide; C = dimethylformamide + ether; D = acetonitrile. [d] Analyses by Weiler and Strauss, Oxford, England. [e] C_3H_5— is allyl. [f] R_1R_2N— is piperidino. [g] R_1 = CH_2—. [h] Isolated as a hydrate. [i] Reported in Ref. 3. [j] R_2 = C_2H_5—.

EXPERIMENTAL

General Procedure for 2-Amino-4-substituted-amino-6-(ureidomethyl)-s-triazines.—To 0.05 mole of the biguanide in 50 ml. of methanol was added 0.05 mole of ethyl hydantoate and the mixture warmed to effect solution. After standing four days at 20° the reaction mixture was added to 50–100 ml. of water. The precipitate of product (see Table I) was separated and recrystallized.

* Received May 17, 1960, from the Research Laboratories of the U. S. Vitamin & Pharmaceutical Corp., Yonkers 1, N. Y.

The authors are indebted to Dr. G. Ungar and his staff for the pharmacological screening of the compounds.

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Note on a Revised Sugar Coating Procedure for Tablets*

By S. J. TUCKER and A. B. REDNICK

A method is presented which describes a revised sugar coating procedure. The procedure eliminates the rounding and smoothing operations usually employed in pan-coated tablets. The resulting coating is approximately one-half as thick as the standard method and coating time is greatly reduced.

TUCKER (1) described the use of insoluble colorants in the sugar coating of tablets. The method has two distinct advantages over the use of water-soluble dyes. The first is that the tablets cannot be overcolored, thus one does not have to be overly concerned about the number of color coats applied. The second is that the color does not have to be applied to perfectly smoothed tablets in order to achieve uniform distribution. These points led us to critically re-evaluate our present sugar coating methods.

In the standard sugar coating procedure a large part of the time is spent in sealing, rounding, and smoothing the tablet and in overcoming the white background during the coloring phase. Also, in the standard coating procedure, the operator must first go to the trouble of compressing a smooth tablet, must then rough it up in order to round the edges, and, finally, must smooth it out before color can be applied.

All these points were taken into consideration when revising the coating procedure employing pigments. The new procedure is divided into three basic phases: (*a*) The tablet is given two coats of acacia, using gelatin solution as the adhesive. This gives the tablet sufficient strength to withstand chipping and cracking in the coating pan and at the same time removes the sharp edges of the tablet. In addition, a colorant is added to the adhesive, thus, coloring begins immediately. (*b*) The tablet is sealed with one coat of a gum or resin cast from a suitable solvent. (*c*) The tablet is finished off using a pigment coating suspension.

DEVELOPMENT AND PROCEDURE

A stock suspension (Table I) is prepared by combining the water, dioctyl sodium sulfosuccinate, and pigment in a Waring Blendor for fifteen minutes. This is then transferred to an Eppenbach Homomixer and, while mixing, the syrup and titanium dioxide are added. The solids usually disperse completely within fifteen minutes.

The stock suspension is then combined with gelatin adhesive solution and mixed with an Eppenbach Homomixer to form the undercoating adhesive suspension (Table II).

TABLE I.—TYPICAL COATING FORMULATION

Stock suspension, ingredients	
Purified water	250.00 ml.
Dioctyl sodium sulfosuccinate	0.01 Gm.
Insoluble colorant	1–15.00 Gm.
Titanium dioxide U. S. P.	0–100.00 Gm.
Syrup U. S. P.	500.00 ml.
Coating suspension	
Stock suspension	100.00 Gm.
Coating syrup *q. s.* ad.	500.00 ml.

TABLE II.—UNDERCOATING FORMULATIONS

Gelatin adhesive solution	
Gelatin U. S. P.	800 Gm.
Purified water	6500 ml.
Acacia U. S. P.	600 Gm.
Sucrose U. S. P.	9500 Gm.
Undercoating adhesive suspension	
Stock suspension	10–20%
Gelatin adhesive solution	80–90%

The core tablets, to be coated, are placed in a conventional coating pan, the size depending upon the batch load. Any standard shaped tablet is suitable (except a flat face); however, a more spherical tablet and a thinner edged tablet facilitate the procedure. Undercoating adhesive suspension is then applied to the rolling tablets until they are evenly and completely wetted. When the mass becomes tacky, acacia powder is dusted on the tablets in the usual fashion. This procedure is repeated again. The tablets now have a color somewhat similar to the finished color, though mottled in appearance. It is interesting to note here that lakes give a definite color to the tablets at this stage, whereas a pigment will not. In order to overcome this, one may use, with the undercoating adhesive, a dye or a lake that gives a similar color (it is not necessary to duplicate the color) to the finished product.

The tablets are now sealed with a gum or resin cast from a suitable solvent. For example, a four-pound cut of shellac diluted with an equal quantity of isopropyl alcohol is satisfactory. One coat is usually sufficient.

Coating suspension (Table I) is now applied and, generally, 25 coats are sufficient. The tablets are finished off and polished in the usual fashion.

The finished product retains the same pharmaceutical elegance as when made by our present methods, yet has a coating of approximately one-half the thickness, and this procedure is accomplished in about one-half the time.

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Book Notices

General Chemistry—Inorganic and Organic. By GARTH L. LEE and HARRIS O. VAN ORDEN. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1960. vi + 637 pp. 15.5 × 23.5 cm. Price $7.25.

This book covers a wide field in simple, concise, positive statements. It could be useful as a textbook in the general science course. The author might have omitted the use of 2 stacked nickels to represent 1 cubic centimeter in the illustration of volume apparatus. It might be difficult for a chemistry minor to grasp the full text, but if he learned to use the book as a starting reference it could be stimulating. A good index is appended and the 1958 revision of atomic weights is included.

Metal-Binding in Medicine. Edited by MARVIN J. SEVEN and L. AUDREY JOHNSON. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. xiii + 400 pp. 18 × 25.5 cm. Price $13.75.

This book includes the papers and panel discussions from a symposium held May 6–8, 1959, in Philadelphia. The term "metal-binding" is used to indicate linkage between a binding agent and a metal, with "chelation" being reserved for the process of metal-binding in which the metal is incorporated into a ring structure. The material covers a wide field, starting with "The Relationship of Chemical Structure to Metal-Binding Action" and including pharmacological, physiological, clinical, and therapeutic considerations of natural and induced metal-binding forms in normal and pathologic systems. An index is appended.

Synthetic Methods of Organic Chemistry. Vol. 14. By W. THEILHEIMER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xvi + 549 pp. 15 × 23 cm. Price $29.50.

Most of the references in Vol. 14 of this compilation of new methods for the synthesis of organic compounds and improvements of known methods cover papers published between 1957 and 1959. A comprehensive and concise reference series.

An Introduction to the Chemistry of Heterocyclic Compounds. By R. M. ACHESON. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xiv + 342 pp. 15 × 23 cm. Price $5.

This book attempts to present to the undergraduate student a concise account of the more important properties and chemical reactions of the basic heterocyclic systems. It is admittedly incomplete, omitting sugars and alkaloids, which the author assumes have been covered in other courses. Included are pertinent physical data, electronic and mechanistic concepts where possible, and brief discussions of chemical discoveries concerning metabolism and biosynthesis of some important compounds. Compound and subject indexes are appended.

Einfache Versuche auf dem Gebiete der organischen Chemie. 8th ed. By A. F. HOLLEMAN and LEONHARD SCHULER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xx + 172 pp. 14 × 22 cm. Paperbound.

This book is a teaching text (in German) of simple and basic chemical reactions of organic compounds and their functional groups.

Analytische Trennung und Identifizierung organischer Substanzen. By OTTO NEUNHOEFFER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xii + 116 pp. 17.5 × 23.5 cm. Price DM 18.

This book (in German) covers concisely the methods for analytical separation and identification of organic substances as applied in a teaching laboratory.

Lehrbuch der organischen Chemie. 35–36 ed. Edited by A. F. HOLLEMAN and FRIEDRICH RICHTER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xii + 646 pp. 17 × 24 cm. Price DM 28.

This classic textbook (in German) has been revised and edited again by Friedrich Richter who has performed this task for at least 30 years. Its reference value has been long established in the U. S. and elsewhere.

Methods in Medical Research. Vol. 8. Edited by H. D. BRUNER. The Year Book Publishers, Inc., 200 East Illinois St., Chicago, Ill., 1960. xiv + 368 pp. 13.5 × 21.5 cm. Price $9.75.

The subjects considered in the eighth volume of this series are grouped under the main headings: Life history of the erythrocyte, Measurement of responses of involuntary muscle, and Peripheral blood flow measurement. Subject and name indexes are appended.

Selective Toxicity. By ADRIEN ALBERT. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. x + 233 pp. 14 × 21.5 cm.

Beginning with a discussion of the biochemical differences between species which introduce the possibility of selectivity into toxicity, four introductory paragraphs of this text are designed for undergraduate students in biochemistry, medicine, pharmacy, and agriculture. Nine other chapters take up advanced material related to biologically-active substances, including absorption and distribution, pharmacodynamics, covalency, surface chemistry, and steric factors. Appendix 1 is a tabulation showing the connection between ionization and antibacterial activity in the acridine series (101 compounds). Appendix 2 tabulates calculated percentage ionized, given pKa and pH (if anion or cation). Appendix 3 lists physical effects of organic substituents.

Medicinal Chemistry. 2nd ed. Edited by ALFRED BURGER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xiii + 1243 pp. 17.5 × 25 cm. Price $37.50.

In the revision of his excellent treatise on medicinal chemistry, Professor Burger has enlisted the aid of 34 contributors. This was done in order to complete a comprehensive revision before portions of the material became out-dated. The expansion of the subject since the first edition of 1951 is indicated by an increase of 12 new chapters. The book is still designed for the more advanced reader and to point the direction of research needed in medicinal chemistry. The thoroughly splendid development and presentation of the text material follows the standard set in the two volumes of the first edition which were reviewed in THIS JOURNAL, **40,** 167, 417(1951). A new two-column format and slightly larger pages have enabled the increased material of the second edition to be published in one volume. The reference value of the book is enhanced by the adequate subject index.

Pharmacology and Therapeutics. 4th ed. By ARTHUR GROLLMAN. Lea & Febiger, Washington Square, Philadelphia 6, Pa., 1960. 1079 pp. 15 × 23.5 cm. Price $12.50.

Publication of the fourth edition of this book only two years after the third edition indicates the author's attempt to keep pace with the rapid changes, especially in therapeutics and drugs. Included are new sections on corticosteroid therapy, treatment of cancer, present-day use and understanding of tranquilizers, the newer hormones, and psychic energizers. All the drugs introduced during the two years prior to the revision of the text are included. Designations of official preparations refer to U. S. P. XV and N. F. X. It is a useful reference and textbook.

Oxidation-Reduction Potentials of Organic Systems. By W. MANSFIELD CLARK. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1960. xi + 584 pp. 15 × 23 cm. Price $13.50.

In referring to the usefulness of the material in this book, the author states: "It should be clearly understood that when concepts of mechanisms and structures are used the purpose is to provide a neat *organization* of relations; confirmation of what is predicted by the final equations does not necessarily prove the postulates." More than 100 tables of data are included in this trustworthy text.

Index Chemicus. Edited by EUGENE GARFIELD. Institute for Scientific Information, 1122 Spring Garden St., Philadelphia 23, Pa., 1960. 131 pp. 21.5 × 28 cm. Paperbound. Issued monthly. Price $250 per year Educational, $500 per year Industrial.

A new (Vol. 1, No. 1) monthly index of new chemicals (3518 in this issue) includes listings of chemical names, structural diagrams, molecular formulas, and complete bibliographical references. Indexes will be cumulated quarterly and yearly.

Recent Progress in Hormone Research. Vol. 16. Edited by GREGORY PINCUS. Academic Press Inc., 111 Fifth Ave., New York 3, N. Y., 1960. viii + 608 pp. 15 × 23 cm. Price $14.

Reports on recent investigations presented at the 1959 Laurentian Hormone Conference are grouped under the main headings: Mechanisms of hormone action, Peptide hormones, Thyroid hormones, and Hormones and metabolism. Discussions of reports are included and author and subject indexes are appended.

Précis de Chimie Biologique. Vol. 2. By J. E. COURTOIS and R. PERLÈS. Masson et Cie., 120, boulevard Saint-Germain, Paris 6e, France, 1960. viii + 620 pp. 16 × 21 cm. Price 46 NF.

Subjects covered in Vol. 2 of this textbook are: Lipides, Proteins, and Experimental functional exploration. A notice on Vol. 1 appeared in THIS JOURNAL, **49,** 182(1960).

Galenisches Praktikum. By KURT MÜNZEL, JAKOB BÜCHI, and OTTO-ERICH SCHULTZ. Wissenschaftliche Verlagsgesellschaft m.b.H., Stuttgart, Germany, 1959. 1114 pp. 16 × 24 cm. Price DM 142.

The "Galenical Handbook" is a textbook (in German) on pharmacy. It is comprehensive in its coverage of procedures, with many examples of formulations included.

The Chemistry of Heterocyclic Compounds. Vol. 14. Pyridine and Its Derivatives. Part I. Edited by ERWIN KLINGSBERG. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. x + 613 pp. 15 × 23 cm. Price $49. Subscription $42.

Part one of a four-part monograph includes: Properties and reactions of pyridine and its hydrogenated derivatives, by R. A. Barnes; and Synthetic and natural sources of the pyridine ring, by F. Brody and P. R. Ruby.

Biochemistry of Plants and Animals. By M. FRANK MALLETTE, PAUL M. ALTHOUSE, and CARL O. CLAGETT. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. xiii + 552 pp. 15 × 23 cm. Price $8.50.

A broad treatment of biochemistry is used to cover a wide variety of topics in the biological, physical, and agricultural sciences at the elementary level. This book replaces "Introduction to Agricultural Biochemistry" by Dutcher, Jensen, and Althouse (Wiley, 1951).

Clinical Chemistry. Principles and Procedures. 2nd ed. By JOSEPH S. ANNINO. Medical Book Department, Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1960. xviii + 348 pp. 15.5 × 23.5 cm. Price $8.

A textbook for students of clinical chemistry on the basic technical level, the major part of the text (pp. 75–339) is devoted to specific methods of clinical analysis.

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